TRAUMATIC BRAIN INJURY: STUDIES ON SERUM BIOMARKERS FOR DIAGNOSIS AND SINGLE NUCLEOTIDE POLYMORPHISMS TO PREDICT OUTCOME

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ABSTRACT

Traumatic brain injury (TBI) is an important cause of morbidity and mortality. The objectives of this study are to determine the links, if any, between selected single nucleotide polymorphisms (SNPs) with 6 month post-TBI outcome, and to identify serum protein biomarkers that may be utilized to detect and grade the severity of TBI.

A cross sectional cohort of 205 hospitalized TBI patients was prospectively studied for the SNPs in the genes of the Apolipoprotein E (*APOE*) and its promoter region, Catechol-O-Methyl Transferase (*COMT*), Dopamine D2 receptor (*DRD2*), Dopamine D3 receptor (*DRD3*), Ciliary Neurotrophic Factor (*CNTF*), Brain Derived Neurotrophic Factor (*BDNF*) and Glial cell Derived Neurotrophic Factor (*GDNF*) using patients blood DNA. The Glasgow Coma Score on admission, and Glasgow Outcome Scale at six months were determined in this cohort and correlated with the findings. A standard iTRAQ protocol was used to identify potential serum protein markers using serum pools from a separate cohort of mild, moderate and severe cases of TBI. Potential peptide candidates were analyzed on a "5800 MALDI TOF/ TOF mass spectrometer" and identified using the "ProteinPilot 4.0" software.

Our analysis showed that possession of at least one *APOE* ε4 allele (OR: 5.5, 95% CI: 2.8-10.7, p= 0.01), *COMT* AA genotype (OR: 18.6, 95% CI: 5.97-58.03, p= 0.01) or at least one *CNTF* A allele (OR: 3.7, 95% CI: 1.88-7.36, p= 0.01) were significantly associated with unfavorable outcome at 6 months post-TBI. In the logistic model, the GCS alone significantly predicted unfavorable outcome in our TBI cohort (R^2 = 30.8, p=0.01). However, GCS paired with the status of *APOE* ε4 allele (R^2 = 60.9; *APOE* ε4 allele- OR: 8.0, 95% CI: 3.6-18.0, p= 0.01; GCS- OR: 5.0, 95% CI: 2.7-9.1, p= 0.01), GCS paired with *COMT* AA genotype (R^2 = 60.61; *COMT* AA genotype- OR: 18.96, 95% CI: 5.48-65.56, p= 0.01; GCS- OR: 3.95, 95% CI: 2.17-7.17, p= 0.01) or GCS paired

with *CNTF* A allele (R^2 = 46.15; *CNTF* A allele- OR: 4.55, 95% CI: 2.10-9.86, p= 0.01; GCS- OR: 4.28, 95% CI: 2.42-7.58, p= 0.01) were better predictors for unfavorable outcome compared with GCS alone. Unfavorable outcome after TBI was not associated with alleles of SNPs of the *APOE* promoter, *DRD2* gene, *DRD3* gene, *BDNF* gene and *GDNF* gene.

We identified 7 biomarkers to be increased in all grades (mild, moderate and severe) of TBI. Two of these markers ("Serum Amyloid- A" and "C- Reactive Protein") were markers for general injury and inflammation. Five markers appear to be specifically increased in TBI ("Leucine- Rich alpha- 2 Glycoprotein- 1", "Lipopolysaccharide Binding Protein", "Fibronectin", "Vitronectin" and "Alpha- 1 antichymotrypsin"). "Apolipoprotein E" and "Zinc Alpha- 2 glycoprotein" were increased only in severe TBI. Conversely, "Gelsolin" was decreased in all grades of TBI, whereas, "Kininogen" was decreased in moderate and severe TBI. If validated, some or all of these protein markers may aid in the detection of TBI, including mild TBI, in conjunction with other conventional diagnostic methods. Our findings suggest that certain serum protein markers and SNPs may be useful to aid clinicians in the diagnosis and grading of TBI, and for prediction of post-TBI outcome, respectively.

ABSTRAK

Trauma Otak: Pengajian Mengenai Biomarker Serum Untuk Diagnosis Dan Polimorfisme Nukleotida Untuk Meramalkan Pemulihan

Kecedaraan otak merupakan punca utama kesakitan dan kematian. Objektif kajian ini adalah untuk menentukan hubungan antara beberapa polimorfisme nukleotida tunggal dengan pemulihan pada bulan yang keenam dan mengenal pasti penanda protein serum yang boleh digunakan untuk mendiagnosis dan menggred trauma otak.

Kami mengkaji 205 pesakit trauma otak di hospital. Polimorfisme nukleotida tunggal daripada gene Apolipoprotein E (*APOE*) dan promoter, Catechol-O-Methyl Transferase (*COMT*), Dopamine D2 receptor (*DRD2*), Dopamine D3 receptor (*DRD3*), Ciliary Neurotrophic Factor (*CNTF*), Brain Derived Neurotrophic Factor (*BDNF*) dan Glial cell Derived Neurotrophic Factor (*GDNF*) telah digenotype menggunakan DNA yang diekstrak daripada darah pesakit. Glasgow Coma Score (GCS) pada kemasukan, dan Glasgow Outcome Scale pada bulan yang keenam ditentukan; dan perkaitan dengan polimorfisme nukleotida tunggal ditentukan. Teknik iTRAQ telah digunakan untuk mengenal pasti protein serum untuk TBI daripada pesakit trauma otak ringan, sederhana dan serius dan sampel kawalan. Akhirnya, peptida dianalisis pada "5800 MALDI TOF/ TOF mass spectrometer" (Applied Biosystems) dan dikenal pasti menggunakan ProteinPilot 4.0.

Analisis menunjukkan, allele *APOE* ε4, *COMT* AA genotype dan allele *CNTF* A dapat meramal pemulihan pesakit yang mengalami kecederaan otak. Kehadiran sekurang-kurangnya satu allele *APOE* ε4 (OR: 5.5, 95% CI: 2.8-10.7, p= 0.01), *COMT* AA genotype (OR: 18.6, 95% CI: 5.97-58.03, p= 0.01) atau kehadiran sekurang- kurangnya

satu allele *CNTF* A (OR: 3.7, 95% CI: 1.88-7.36, p= 0.01) adalah sangat berkait dengan pesakit yang tidak pulih dengan baik. Dalam model logistik, GCS sendiri dapat meramal samaada seorang pesakit tidak akan pulih dengan baik selepas kecederaan otak (R^2 = 30.8, p=0.01). Tetapi, model tersebut dapat ramal dengan lebih baik sekiranya GCS digabungkan dengan status allele *APOE* ε4 (R^2 = 60.9; Allele *APOE* ε4- OR: 8.0, 95% CI: 3.6-18.0, p= 0.01; GCS- OR: 5.0, 95% CI: 2.7-9.1, p= 0.01), GCS digabungkan dengan *COMT* AA genotype (R^2 = 60.61; *COMT* AA genotype- OR: 18.96, 95% CI: 5.48-65.56, p= 0.01; GCS- OR: 3.95, 95% CI: 2.17-7.17, p= 0.01) atau GCS digabungkan dengan status allele *CNTF* A (R2 = 46.15; Allele *CNTF* A- OR: 4.55, 95% CI: 2.10-9.86, p= 0.01; GCS- OR: 4.28, 95% CI: 2.42-7.58, p= 0.01). Pemulihan yang tidak baik tidak berkaitan dengan polimorfisme nukleotida pada gene *APOE* promoter, gene *DRD2*, gene *DRD3*, gene *BDNF* dan gene *GDNF*.

Kami mengenalpasti 7 biomarker meningkat dalam semua gred kecederaan otak. Dua daripadanya ("Serum Amyloid A" dan "C- Reactive protein") turut meningkat dalam inflamasi dan kecederaan biasa yang lain. Lima biomarker didapati meningkat secara spesifik selapas kecederaan otak ("Leucine Rich alpha-2 Glycoprotein-1", "Lipopolysaccharide Binding Protein", "Fibronectin", "Vitronectin" dan "Alpha- 1 antichymotrypsin"). "Apolipoprotein E" dan "Zinc Alpha- 2 glycoprotein" hanya meningkat dalam kecederaan otak yang serius. Manakala, aras "Gelsolin" dalam serum menurun selepas semua gred kecederaan otak, dan aras "Kininogen" hanya menurun dalam kecederaan tahap sederhana dan serius. Jika disahkan, biomarker- biomarker ini dapat memastikan kewujudan kecederaan otak dalam seseorang individu termasuk kecederaan otak yang ringan, dan dapat mendiagnosis dan menggred kecederaan otak, supplemen kepada kaedah mendiagnosis sedia ada yang lain.

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LIST OF SYMBOLS AND ABBREVIATIONS

<	Smaller than
>	Greater than
≥	Greater than or equal to
≤	Smaller than or equal to
%	Percentage
α	Alpha
β	Beta
ε	Epsilon
A	Adenine
T	Thymine
G	Guanine
C	Cytosine
g	Gram
mg	Milligram
µg	Microgram
ng	Nanogram
L	Liter
mL	milliliter
µL	Microliter
˚C	Degree Celcius
TBI	Traumatic brain injury
SNPs	Single Nucleotide Polymorphisms
GCS	Glasgow Coma Scale

GOS	Glasgow Outcome Scale
ApoE	Apolipoprotein E
APOE	Apolipoprotein E gene
CNS	Central Nervous System
COMT	Catechol-O-Methyl Transferase enzyme
COMT	Catechol-O-Methyl Transferase gene
DRD2	Dopamine D2 receptor gene
DRD3	Dopamine D3 receptor gene
ANKK1	Ankyrin repeat and kinase domain containing 1
BDNF	Brain Derived Neurotrophic Factor
GDNF	Glial cell Derived Neurotrophic Factor
CNTF	Ciliary Neurotrophic Factor
BDNF	Brain Derived Neurotrophic Factor gene
GDNF	Glial cell Derived Neurotrophic Factor gene
CNTF	Ciliary Neurotrophic Factor gene
CT Scan	Computed Tomography Scan
GFAP	Glial fibrillary acidic protein
NSE	Neuron Specific Enolase
MBP	Myelin Basic Protein
FABP	Fatty acid-binding proteins
B-FABP	Brain type fatty acid-binding proteins
H-FABP	Heart type fatty acid-binding proteins
CKBB	Creatine kinase brain isoenzyme
IL	Interleukin
TNF-α	Tumor Necrosis Factor-α
TGF-β	Transforming Growth Factor-β

ICAM-1	Intercellular Adhesion Molecule
MIP	Macrophage inflammatory protein
VWF	Von Willebrand Factor
MMP	Matrix metalloproteinase
HSP	Heat shock proteins
mL	Milliliter
NaCl	Sodium Chloride
PCR	Polymerase Chain Reaction
TE Buffer	Tris- EDTA Buffer
DNA	Deoxyribonucleic Acid
SCX liquid chromatography	Strong Cation Exchange liquid chromatography
HPLC	High Performance Liquid Chromatography
KCl	Potassium Chloride
SSC buffer	Standard Saline Citrate buffer
SDS buffer	Sodium Dodecyl Sulphate buffer
HCl	Hydrocloric Acid
TAE buffer	Tris-acetate-EDTA buffer
EDTA	Ethylenediaminetetraacetic acid
iTRAQ	Isobaric tag for relative and absolute quantitation
MS	Mass spectrometry
MALDI	Matrix-Assisted Laser Desorption Ionization
GWAS	Genome Wide Association Studies

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CHAPTER 1

INTRODUCTION

Traumatic brain injury (TBI) occurs when an external force damages the brain and can result from motor vehicle accidents, from falls or from blunt force assault. TBI is one of the major health and socioeconomic problems in Malaysia and can be considered a "silent epidemic". It is the prime cause of death for people who are below 45 years old of age (Moppett, 2007) and motor vehicle accidents are considered as the major cause of TBI. Based on statistics, the death rate due to motor vehicle accident in Malaysia is one of the highest in the world with approximately 22 deaths per 100, 000 inhabitants (Liew et al., 2009). Currently there are only seven general hospitals and three university hospitals in Malaysia with neurosurgical services to serve the entire population of 30 million. With such inadequate neurosurgical facilities and intensive care units in Malaysia, there is an alarming and critical lack of services to cater for the large number of TBI patients (Liew et al., 2009).

The pathophysiology of TBI is complex, and outcome depends on the type, severity and location of injury. Primary brain injury is induced by direct impact to the brain parenchyma leading to focal or diffuse tissue distortion, destruction, tearing, and/or hemorrhage. As a result of primary brain injury, neurons can die by necrosis caused by membrane disruption, irreversible metabolic disturbances and excitotoxicity (Chesnut et al., 1993). Primary brain injury in the brain can be fatal or give rise to severe disability due to neuronal destruction. A cascade of events, triggered either immediately after TBI or in the following hours or days, could lead to cerebral ischemia, cerebral edema, hematoma formation, hypoxia and hypotension (Chesnut et al., 1993; Marmarou et al., 1991). These conditions are collectively known as secondary brain injury. In contrast to primary brain injury where neuronal death is mainly caused by necrosis, secondary brain

injury is marked mainly by neuronal apoptosis. Neuronal apoptosis involves the Caspase cascade and release of apoptogenic factors from mitochondria and lysosome which leads to systematic fragmentation of cellular DNA, collapse of nuclear structure and cytoskeletal disintegration. This is followed by the formation of membrane wrapped apoptotic bodies (Clark et al., 2001; Clark et al., 1997) and exposure of phosphatidyl-serine on the cell membrane surfaces and subsequently detection and engulfment by microglia (Borisenko et al., 2003).

Expression studies have shown that several genes are involved in the pathophysiology of secondary brain damage. Single nucleotide polymorphisms (SNPs) of various genes have been implicated to dramatically worsen primary damage, leading to the activation of a cascade of neuronal and axonal pathologies, which in turn could impact on the patient's overall clinical outcome. To date, the association between SNPs in the *APOE* gene and its promoter with outcome after TBI have been most extensively studied. Some SNPs of genes involved in dopamine systems, and neurotropic factors may have the potential to influence the outcome after TBI. The associations between outcomes after TBI with these SNP's in our local population have not been studied before. Indeed, there is insufficient data in Asian populations to compare with data derived from Western populations.

There is also considerable interest to develop protein markers that can be used to confirm the presence of TBI in patient with polytrauma and to distinguish and grade the severity (mild, moderate or severe) of TBI. If a reliable protein marker for TBI could be found that appears soon after TBI, is easily measurable in blood, has an absolute value proportional to the extent of brain and has a potential for early prognosis, TBI management could conceivably be improved. Unfortunately, to date an ideal TBI protein marker has yet to be found. Traditional biomarker identification rely on the ELISA method and/or high-resolution protein separation by 2D gels using dyes, fluorophores or

radioactivity to visualize and quantify the relative abundance of proteins (Bantscheff, Lemeer, Savitski, & Kuster, 2012; Schulze & Usadel, 2010). Unfortunately, many samples are required for ELISA, and image analysis of 2D gels is labour intensive and often subjective. Mass spectrometry-based proteomics has emerged as a new technology over the last decade to perform large-scale analysis of proteins, and has become routine in many types of biomarker analysis (Schulze & Usadel, 2010), including TBI (Hergenroeder et al., 2008).

The general objectives of this study are to determine the associations between selected SNPs with outcome after TBI and to identify serum protein biomarkers that can be utilized to diagnose and grade the severity of TBI.

The specific objectives are:

1. To determine the association between polymorphisms in the *APOE* gene and promoter, and outcome after TBI.
2. To determine the association between polymorphisms in the genes modulating dopamine system and outcome after TBI.
3. To determine the association between polymorphisms in the genes modulating neurotropic factors and outcome after TBI.
4. To identify serum biomarkers in mild, moderate and severe TBI by mass spectrometry.
5. To determine the association between serum biomarkers and severity of the TBI.

CHAPTER 2

LITERATURE REVIEW

2.1 Glasgow Coma Scale

Consistent and precise recording of neurological observations is important to establish the patient's neurological status after brain injury. The Glasgow Coma Scale (GCS) was introduced in 1974 with the aim of standardizing the assessment of the level of consciousness in head injury patients (Teasdale & Jennett, 1974). It has been used mainly in evaluating prognosis, comparing different groups of patients and for monitoring the neurological status. Traditionally, GCS based on the clinical presentation on admission, is the gold standard to classify patients into mild, moderate or severe brain injury.

Table 2.1 shows the GCS scores and their definitions. The GCS is divided into three sections: "eye opening", "verbal responses" and "motor responses". The patient is assessed and scored in each area and the scores are then added together to give the patient's GCS score - "The highest possible score is 15; the lowest is 3. A patient who is fully aware and orientated will score 15; a lower score will reflect a lower level of consciousness. It is important to stress that for clinical use, the three separate scores (eye opening, verbal responses and motor responses) rather than the total sum score more effectively reflects the patients' clinical status. A cumulative score of 14-15 is classified as mild TBI, 9-13 as moderate TBI and ≤ 8 as severe brain injury" (Baalen et al., 2003; Teasdale & Jennett, 1974, 1976).

Table 2.1: Glasgow Coma Scale with scores (Baalen et al., 2003; Teasdale & Jennett, 1974, 1976).

Eye opening [1]	
4	"Spontaneous. Indicates arousal, not necessarily awareness"
3	"To speech. When spoken to – not necessarily the command to open eyes"
2	"To pain. Applied to limbs, not face where grimacing can cause closure"
1	"None"
Motor response [2]	
6	"Obeys commands. Exclude grasp reflex or postural adjustments"
5	"Localises. Other limb moves to site of nailbed pressure"
4	"Withdraws. Normal flexion of elbow or knee to local painful stimulus"
3	"Abnormal flexion. Slow withdrawal with pronation of wrist, adduction of shoulder"
2	"Extensor response. Extension of elbow with pronation and adduction"
1	"No movement"
Verbal responses [3]	
5	"Orientated. Knows who, where, when; year, season, month"
4	"Confused conversation. Attends & responds but answers muddled/wrong"
3	"Inappropriate words. Intelligible words but mostly expletives or random"
2	"Incomprehensible speech. Moans and groans only – no words"
1	"None"

[1] "Eye opening- this should be spontaneous when the patient is approached. If the patient's eyes do not open spontaneously, determine whether they open to speech or painful stimuli. If the eyes are open, do not assume that the patient is fully aware and orientated to their surroundings. Some patients with head injuries may have spontaneous blinking and eye movement; this does not indicate full consciousness."

Table 2.1, Continued: Glasgow Coma Scale with scores (Baalen et al., 2003; Teasdale & Jennett, 1974, 1976).

[2] "Motor response- this is assessed by giving the patient some simple commands, for example 'squeeze my hand (both sides)', 'lift your legs up off the bed', and 'show your tongue'. The strength of the patient's limbs should be noted- it is essential to observe for weaknesses. When a patient does not respond to simple commands then response to painful stimuli is assessed. The practice of pinching the patient and/or using a pinprick test is no longer recommended for assessing motor response and should not be used during a neurological evaluation. There are three recognised stimuli that can be used when assessing motor response:

a. Trapezium squeeze- use the thumb and two fingers to squeeze and twist the muscle;
b. Supraorbital pressure- run a finger along the bony rim above the eye. This is not recommended if the patient has facial fractures;
c. Sternal rub- using the knuckles of a clenched fist, apply pressure along the sternum."

[3] "Verbal response- this determines state of consciousness. Patients who are fully orientated will know their name, where they are, the date and year. A confused patient may be able to hold a conversation, but when asked questions may give replies which are incorrect or inapt. They may use inappropriate words, which do not make sense to the assessor, or they may make incomprehensible sounds such as moans or groans. Some stimuli may be required to obtain a response from the patient- this type of patient is not aware of their surroundings"

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After four decades, the GCS is now internationally recognized as an efficient assessment of consciousness. The scale progressively occupied a central role in clinical guidelines and an integral component of scoring systems for patients with TBI (G. Teasdale et al., 2014).

2.2 Glasgow Outcome Scale

Head injury is associated with significant morbidity and mortality. The Glasgow Outcome Scale (GOS) is one of several ways of measuring the outcome after head injury (Jennett & Bond, 1975). Table 2.2 shows the GOS scales and their definitions. It is a global scale for functional outcome that groups patient status into one of five categories: "Dead, Persistent vegetative state, Severe disability, Moderate disability or Good recovery". The GOS is the most widely used and accepted measure of outcome following head injury and provides an overall assessment suitable for the comparison of outcomes (Wade, 1992). It is a simple, reliable means of describing recovery (Jennett & Bond, 1975) that is quick to administer, broadly applicable and has clinically relevant categories (Wilson, Pettigrew, & Teasdale, 2000). It has been adopted widely for use in clinical trials (Hellawell, Signorini, & Pentland, 2000; Wade, 1992; Wilson et al., 2000).

Table 2.2: Glasgow Outcome Scale (Jennett & Bond, 1975)

1	Death	"dead"
2	Persistent vegetative state	"wakefulness without awareness; absence of speech or evidence of mental function in a patient who appears awake with spontaneous eye opening"
3	Severe disability	"conscious but dependent; patient requires assistance to perform daily activities and cannot live independently"
4	Moderate disability	"independent but disabled; patient unable to return to work but otherwise able to independently perform the activities of daily living"
5	Good recovery	"reintegrated but may have non disabling sequelae; able to return to work but not necessarily at the same level; may have minor neurological or psychological impairments"

When an evaluation is to be done mainly depends on the aim for which it is being conducted. If the evaluation is done to measure mortality, it should be assessed at the time of discharge, since most deaths occur in the first week. Since some patients continue to improve over the years, it has to be noted that estimation of ultimate outcome might be tedious. Continuing a follow-up over years is very tedious and impractical to evaluate outcome of an acute condition. Studies using a 5-point outcome scale have shown that most patients reached their final point by six months, although it is undeniable that small number of patients will continue to improve within a category (Teasdale, 1982). Thus, most international studies emphasises outcome evaluation at 6 month.

The assignment of an individual to an outcome category should be based on the results of a structured interview focused on social and personal functional abilities (Jennett & Bond, 1975). An interrater reliability study of the structured interview for the GOS yielded a weighted kappa value of 0.89. Hence, GOS assessment using a standard format with a written protocol is practical and reliable (Wilson, Pettigrew, & Teasdale, 1998). This has increased the reliability of postal and telephone administration to determine a GOS rating (Wilson et al., 2002). Each interview incorporates a means for including information regarding pre-injury status, thereby providing a means for determining the effect of the sequelae of head injury on outcome separate from the effects of pre-existing conditions or circumstances (Pettigrew, Wilson, & Teasdale, 1998; Wilson et al., 1998). The final rating is based on the lowest category of outcome indication in the interview (Wilson et al., 2000).

2.3 Genetic and serum protein biomarkers for TBI

Interest for genetic and serum biomarkers in TBI had been rapidly increasing in the past several decades. Mainly because individuals with seemingly very similar injuries often have vastly different outcomes, it is believed that genetic factors may explain, in part, different recovery trajectories. As genetic and genomic technologies have improved, genetic analysis has become more accessible and increasingly used to study outcomes after TBI. SNPs are genetic variants that arises from a difference in a single base pair in the deoxyribonucleic acid. SNPs may be located in critical areas in genes and so may ultimately lead to changes in proteins that may be important in certain disease specific pathways. On the other hand, serum protein markers of TBI are also gaining interest. Serum protein markers are protein molecules which may be increased or decreased as a response to TBI. Alterations in levels of serum proteins within 24 hours in patients with

mild, moderate and severe TBI may be useful as an adjunct to detect and even grade TBI, especially in the polytrauma setting. Especially in mild TBI, as the patients' GCS scores are normal, and neuroimaging investigations such as the CT scan or brain MRI may not show evidence of injury. These biomarkers allow a quantitative diagnostic, even for patients who cannot be evaluated by GCS due to intubation, ventilation and/or sedation.

Although quite a number of research studies had been conducted, the effort to identify good genetic and serum protein markers still goes on. Confounding factors include varying degrees of secondary damage to the brain after the primary injury. The location and extent of tissue damage may not correspond with outcome because a small area of damage to an eloquent area could give rise to a more unfavorable outcome compared to a larger area of injury to a less critical area. In addition, restriction of brain tissue proteins flowing into the circulation by the blood brain barrier, and combination of TBI with trauma to extracranial organs increases the difficulty to find a sensitive and specific protein marker for TBI (Feala et al., 2013; Mondello et al., 2011).

2.4 SNPs as biomarkers of TBI

The majority of genetic biomarker studies in TBI have been 'candidate SNP association studies'. Expression studies had shown that numerous genes are associated in the pathophysiology of TBI. The SNPs of *APOE* gene and its promoter, candidate SNPs of dopamine systems and candidate SNPs of neurotropic factors are postulated to prospectively influence TBI outcome. Studying SNPs offers hope for the possible development of new patient management and therapeutic strategies.

2.4.1 Apolipoprotein E (ApoE)

ApoE is a 299 amino acid protein with a relative molecular mass of 34 KDa. ApoE is the major apolipoprotein in the human cerebrospinal fluid. It exists as small spherical, discoidal lipoprotein, often packed together with cholesterol and phospholipid to form lipid-protein complexes, which are then released into the extracellular space. These complexes bind to ApoE receptors on the surfaces of nerve cells and are internalised into the cell.

In the peripheral nervous system, ApoE is synthesized primarily by the glia cells surrounding motor and sensory neurons, and in non-myelinated Schwann cells. Large quantities of ApoE are also secreted by resident macrophages and those recruited to injured peripheral nerves, which accumulates in the extracellular matrix of the regenerating nerve and degenerating stump (Mahley, 1988). It was initially observed that ApoE concentration increases 200-fold in an injured rat sciatic nerve (Boyles et al., 1989; Ignatius et al., 1986; Ignatius, Shooter, Pitas, & Mahley, 1987) and then returns to baseline by 8 weeks when sciatic nerve regeneration was largely complete. Studies of peripheral nerve injury in ApoE -/- mice suggested that normal regeneration can occur

without ApoE (Popko, Goodrum, Bouldin, Zhang, & Maeda, 1993). However, ultrastructural examination revealed a reduced number of axons, and defects in their morphology (Fullerton, Strittmatter, & Matthew, 1998).

In the central nervous system (CNS), astrocytes are the major cells that produce ApoE (Boyles et al., 1985; Pitas et al., 1987). However, under pathological and physiological conditions, CNS neurons also express ApoE (Bao et al., 1996; Beffert & Poirier, 1996; Diedrich et al., 1991; Han et al., 1994; Harris et al., 2004; Metzger et al., 1996; Xu et al., 1999; Xu et al., 1998; Xu, Schmechel, et al., 1999; Xu et al., 1996). Treatment with kainic acid induces ApoE synthesis in hippocampal neurons in rats (Boschert, Merlo-Pich, Higgins, Roses, & Catsicas, 1999), and ApoE is expressed in neurons in patients with cerebral infarcts (Aoki et al., 2003). It has been postulated that neuronal expression of ApoE is induced to promote neuron repair or protection (Huang et al., 2004; Mahley, 1988; Mahley & Huang, 1999; Mahley & Rall, 2000; Weisgraber, 1994).

2.4.1.1 ApoE isoforms and effects

The *APOE* gene spans 3.7 kilobases, has four exons, and is located on the long arm (q) of chromosome 19 at position 13.2 (19q13.2) (Houlston, Snowden, Green, Alberti, & Humphries, 1989). ApoE are encoded by three alleles (ε2, ε3 and ε4) of the *APOE* gene (Roses, 1996) giving rise to 3 homozygous genotypes (*APOE* ε2/ε2, *APOE* ε3/ε3, and *APOE* ε4/ε4) and three heterozygous genotypes (*APOE* ε2/ε3, *APOE* ε3/ε4, and *APOE* ε2/ε4). These three alleles arise due to SNPs at positions 112 and 158. The *APOE* ε2 allele have TGCs at both positions, *APOE* ε4 allele have CGCs at both positions, whereas *APOE* ε3 allele has TGC at position 112 and CGC at position 158, respectively.

Due to the SNPs, ApoE has three common isoforms (E2, E3 and E4): ApoE E2 (cysteine at both positions), ApoE E4 (arginine at both positions), whereas ApoE E3 has cysteine at position 112 and arginine at position 158, respectively. These small changes between the isoforms cause significant alterations in structure and function.

ApoE has two structural domains: "a 22-kDa N-terminal domain (residues 1–191) containing the low-density lipoprotein receptor binding site (residues 136–150) and a 10-kDa C-terminal domain (residues 216–299) containing the major lipid binding site (residues 240–270) (Mahley, 1988; Mahley & Huang, 1999; Mahley & Rall, 2000; Weisgraber, 1994; Weisgraber & Mahley, 1996). In ApoE E4, but not to the same extent as in ApoE E2 or ApoE E3, the two domains interact (Dong & Weisgraber, 1996; Dong et al., 1994). X-ray crystallographic studies indicate that arginine-112 in ApoE E4 allows the side chain of arginine-61 to extend away from the helical bundle (Dong et al., 1994) and interacts with glutamate-255 in the C-terminal region (Weisgraber, 1990). The side chain of arginine-61 in ApoE E3 and ApoE E2 has a different orientation (tucked between helices 2 and 3) (Wilson et al., 1991) and are much less likely to undergo domain interaction. Because of this, ApoE E4 is the least stable isoform. Furthermore, the denaturation pattern of ApoE E4 does not fit a two-state equilibrium (native versus fully unfolded), suggesting that ApoE E4 exists as a partially folded intermediate or a molten globule. These reactive intermediates have several pathophysiological activities (Dobson, 2001; Ptitsyn, 1995), including altered intradomain interactions, increased lipid and membrane binding, membrane disruption, translocation across membranes, and increased susceptibility to proteolysis".

Consequently, ApoE E3 (and ApoE E2) seems to be more effective in the normal maintenance and repair of cells than ApoE E4, which may be in fact be detrimental to the process. Isoform-specific effects of ApoE have been demonstrated on neurite extension culture systems (Bellosta et al., 1995; DeMattos, Curtiss, & Williams, 1998; Holtzman et

al., 1995; Nathan et al., 1994; Sun et al., 1998). In the presence of a source of lipid, ApoE E3 stimulates neurite outgrowth, whereas ApoE E4 does not. Astrocyte-derived ApoE E3, but not ApoE E4, also caused neurite extension in rat hippocampal neurons (Sun et al., 1998). ApoE E4 inhibition of neurite extension appears to be related to alterations in the cytoskeleton, that impacts on microtubule stability (Nathan et al., 1995). These effects may be mediated through tau (a microtubule stabilizing protein). ApoE E3, but not ApoE E4, binds to tau in vitro, and may protect tau from hyperphosphorylation, a process which inhibits the ability of tau to stabilize microtubules (Lovestone et al., 1996; Strittmatter et al., 1994; Tesseur et al., 2000).

2.4.1.2 TBI and the *APOE* ε4 allele

Despite great efforts to investigate the role of *APOE* gene on outcome after TBI, in most of these studies, its role remains equivocal. We believe it may be due to sample populations and varying prevalence of *APOE* ε4 allele in the different study populations. Besides, in most studies, there is a case mix of mild to severe TBI patients (Chamelian, Reis, & Feinstein, 2004a; Pruthi et al., 2010), that could explain the divergent results of *APOE* polymorphism influence on TBI outcome. In order to generate robust data, a much larger sample is required, which might not be realistic.

Teasdale et al. (1997) in a prospective evaluation of 89 patients with TBI, observed a 2-fold increase in unfavorable outcome at 6 months with 17 of 30 (57%) patients with *APOE* ε4 allele who had unfavorable outcome compared with only 16 of 59 patients (27%) without *APOE* ε4 allele. “Death”, “vegetative state”, or “severe disability” was defined as unfavorable outcome using the GOS (G. M. Teasdale, Nicoll, Murray, & Fiddes, 1997). Several other clinical studies in adults with TBI support the notion that *APOE* ε4 allele is associated with a poor prognosis and unfavorable outcome (Chiang,

Chang, & Hu, 2003; Friedman et al., 1999; Liaquat, Dunn, Nicoll, Teasdale, & Norrie, 2002) (Table 2.3). Most of these reports document the association between *APOE* ε4 allele and post-TBI outcome almost exclusively in Caucasian or white populations. Other studies suggested that *APOE* ε4 allele has no impact on recovery from TBI (Alexander et al., 2007; Chamelian et al., 2004a; Diaz-Arrastia et al., 2003; Millar, Nicoll, Thornhill, Murray, & Teasdale, 2003; Nathoo, Chetry, van Dellen, Connolly, & Naidoo, 2003; Pruthi et al., 2010; G. M. Teasdale, Murray, & Nicoll, 2005; Willemse-van Son, Ribbers, Hop, van Duijn, & Stam, 2008). In a South African black population with a relative high frequency of *APOE* ε4 allele, *APOE* ε4 allele was not found to have a significant effect on TBI outcome (Nathoo et al., 2003). Yet other studies showed insignificant association between *APOE* genotype with outcome in mild TBI (Chamelian et al., 2004a; Sundstrom et al., 2004). Thus, further research to study the association between TBI outcome and *APOE* polymorphism is needed, particularly in non-caucasian racial groups.

Table 2.3: Baseline variables of studies on the association between *APOE* genotype and TBI outcome

Author and Year	Country	Outcome Evaluation Method (Blinded to *APOE* genotype)	Sample Size	GCS			OUTCOME			
							Non *APOE*ε4 allele carrier		*APOE*ε4 allele Carrier	
				Mild	Moderate	Severe	Unfavorable	Favorable	Unfavorable	Favorable
(G. M. Teasdale et al., 1997)	United Kingdom	Direct Interviews (Yes)	89	48	19	22	16	43	17	13
(Friedman et al., 1999)	Israel	Direct Interviews (Yes)	69	X	X	X	29	13	26	1
(Liaquat et al., 2002)	United Kingdom	No Info (Yes)	129	53	26	50	92 patients, 21% have poor outcome (~19)		37 patients, 43% with poor outcome (~16)	
(Diaz-Arrastia et al., 2003)	South Africa	Phone interviews (Yes)	106	58	13	35	10	19	21	56
(Millar et al., 2003)	United Kingdom	No Info (Yes)	396	27	21	272	73	206	28	88
(Chiang et al., 2003)	Taiwan	Direct Interviews (Yes)	100	36	24	40	20	61	10	9
(Nathoo et al., 2003)	United States of America	No Info (Yes)	110	59	31	20	10	55	11	34

Table 2.3, Continued: Baseline variables of studies on the association between *APOE* genotype and TBI outcome

Author and Year	Country	Outcome Evaluation Method (Blinded to *APOE* genotype)	Sample Size	GCS			OUTCOME			
							Non *APOE*ε4 allele carrier		*APOE*ε4 allele Carrier	
				Mild	Moderate	Severe	Unfavorable	Favorable	Unfavorable	Favorable
(Chamelian et al., 2004a)	Canada	No Info (Yes)	90	X	X	X	X	X	X	X
(G. M. Teasdale et al., 2005)	United Kingdom	Direct Interviews (Yes)	984	513	178	267	215	445	118	206
(Alexander et al., 2007)	United States of America	Direct Interviews (Yes)	123	X	X	X	97 without *APOE* E4 allele		26 with *APOE* E4 allele	
(Willemse-van Son et al., 2008)	Netherlands	Direct Interviews (Yes)	79	X	X	X	13	43	2	12
(Pruthi et al., 2010)	India	Direct Interviews (Yes)	73	X	X	X	11	50	0	12

2.4.1.3 *APOE* Promoter

Three SNPs in the *APOE* promoter at positions -491 A/T, -427 C/T, and -219 G/T (also known as Th1/E47cs) have been variously reported to alter transcriptional activity of the *APOE* promoter due to differential binding of transcription factors (Artiga et al., 1998; Lambert et al., 1998; Lambert et al., 1998). The -491T allele is associated with a diminished transcription, -427 polymorphism with an unaltered transcription and -219G allele with an enhanced transcription. The various allelic combinations for each position are: -491 AA or -491 AT or -491 TT; -427 CC or -427 CT or -427 TT; -219 GG or -219 GT or -219 TT. These SNPs are also speculated to influence the outcome after TBI. Lendon et al. (2003) noted that poor recovery from TBI was more frequent among subjects with the TT genotype of -219 G/T compared to the GG and GT genotypes. However, no association was noted between unfavorable outcome and -491 A/T promoter polymorphism (Lendon et al., 2003). To date there is no study on -427 C/T promoter polymorphisms and outcome.

2.4.2 SNPs in genes modulating the dopamine system

Dopamine is an endogenous neurotransmitter that belongs to the catecholamine and phenethylamine families. When released at the presynaptic site of a neuron, it carries the signal through the synaptic cleft to reach the postsynaptic site of another neuron where it binds to dopamine receptors (mainly D1 to D5). Dopamine is the major neurotransmitter in the central dopaminergic pathways that plays an important role in motor function (the nigro-striatal pathway), motivated behavior, hormonal stasis (the tubero-infundibular pathway), cognition (meso-cortical pathway), mood homeostasis and reward circuitry (the meso-limbic pathway). It may also be involved in neural plasticity

and repair through effects on the brain derived neurotrophic factor (Guillin et al., 2004), and in recovery of motor function after TBI as seen in animal and human studies (Martinsson & Eksborg, 2004).

The level of dopamine is carefully regulated in the central dopaminergic system. Moreover, dopaminergic systems are susceptible to injury and TBI associated dysregulation (Kobori, Clifton, & Dash, 2006; McAllister, Flashman, Sparling, & Saykin, 2004) leads to increased level of dopamine, which is a potent excitotoxin to neurons. High levels of dopamine in the synaptic cleft are rapidly oxidized to form dopamine semiquinone/quinine. Oxidized dopamine *via* monoamine oxidase activity or redox cycling can induce the generation of hydrogen peroxide and superoxide that cause significant oxidative stress (Brunmark & Cadenas, 1988; Hastings, 1995; Olney et al., 1990; Sinet, Heikkila, & Cohen, 1980; Williams & Castner, 2006). Furthermore, dopamine signaling at the dopamine D2 receptor can induce increases in intracellular Ca2+ release and activation of calcium dependent kinases and phosphatases important for cell death signaling (Azdad et al., 2009; Hernandez-Lopez et al., 2000; So et al., 2009).

Many genetic studies have focused on SNPs in genes that modulate the dopamine system in neuropsychiatric disorders such as Parkinson's disease, and treatment of schizophrenia with neuroleptics. The candidate SNPs that have been investigated include rs4680 (G/A) polymorphism in the Catechol-O-Methyl Transferase gene, rs1800497 (C/T) polymorphism of the Dopamine D2 receptor gene and rs6280 (C/T) polymorphism in the dopamine D3 receptor gene. Relatively little work has been dedicated to correlating these SNPs with outcomes in TBI.

2.4.2.1 Catechol-O-Methyl Transferase

Catechol-O-Methyl Transferase (COMT) is an enzyme involved in the catabolism of dopamine through methylation of dopamine and norepinephrine. The human *COMT* gene is located on chromosome 22q11.21–23. The rs4680 SNP in the *COMT* gene results in a change from G to A at position 472. The G allele codes for valine and A allele codes for methionine. The enzyme with valine is almost four times as active as methionine at normal body temperature. Under conditions of increased dopamine release, individuals with *COMT* A alleles may have less efficient neurotransmission and worse performance (Egan et al., 2001; Lachman et al., 1996; Syvanen, Tilgmann, Rinne, & Ulmanen, 1997). To date there is no study of the association of this SNP with functional outcome after TBI. However, a study which evaluated cognitive performance of 130 (indicate poor cognitive performance) individuals with TBI showed that GG homozygotes made more perseverative responses on the "Wisconsin Card Sorting Test", while AA homozygotes had the least number of perseverative responses (Lipsky et al., 2005).

2.4.2.2 Dopamine D2 receptor

The human Dopamine D2 receptor (*DRD2*) gene is located on chromosome 11q22–23, and consists of eight exons. Until 2004, rs1800497 which is a C to T SNP was thought to lie in a regulatory region of DRD2. The *DRD2* T allele has been associated with a 40% decrease in the D2 receptors expression (Pohjalainen et al., 1998). In 2004, Neville et al. (2004) mapped rs1800497 to the last exon in the adjacent gene *ANKK1*. *ANKK1* is a novel member of the serine/threonine kinase gene family with 11 ankyrin repeats (Neville, Johnstone, & Walton, 2004). Ankyrin repeats are involved in widely diverse processes, including protein-protein interactions and transcription initiation

(Hryniewicz-Jankowska, Czogalla, Bok, & Sikorsk, 2002; Lubman, Korolev, & Kopan, 2004). It is likely that the *ANKK1* gene is involved directly in dopaminergic signaling or in DRD2 transcription. To date there is no study which associates this SNP with functional outcome after TBI. However, a study by McAllister et al. (2005) of 39 patients with mild TBI showed that the T allele is associated with poorer performance in California Verbal Learning Test recognition task and the Continuous Performance Test. The study concluded that the T allele can affect cognitive outcome following mild TBI (McAllister et al., 2005).

2.4.2.3 Dopamine D3 receptor

The Dopamine D3 receptor (*DRD3*) gene is located on chromosome 3q13.3, and its coding sequence consists of six exons. The rs6280SNP in the *DRD3* gene results in a change from C to T. Studies have shown that *DRD3* C (glysine) confers an increased affinity for dopamine compared to *DRD3* T (serine) (Wong, Buckle, & Van Tol, 2000). However, it is not known whether it affects outcome after TBI.

2.4.3 SNPs in genes of neurotrophic factors

Neurotrophic factors are small protein molecules that regulate growth and survival of neurons, associated with metabolic functions such as protein synthesis and synthesis of neurotransmitters that carry chemical signals which allow the neuron to communicate with other neurons or with other targets (muscles, glands, etc.). Because of these actions, neurotrophic factors play a significant role in the maintenance of neuronal function throughout an individual's entire lifetime (Huang & Reichardt, 2001; Thoenen, 1995).

Neurotrophic factors have been involved in the pathogenesis of some neurodegenerative disorders, and some factors have been proposed as potential treatments for these diseases on the basis of in vitro experiments and animal model studies (Moris & Vega, 2003). Many have studied brain-derived neurotrophic factor, glial cell-derived neurotrophic factor and ciliary neurotrophic factor on neuropsychiatric disorders such as Alzheimers's disease, and in the treatment of schizophrenia (Huang et al., 2007; Lavedan, Volpi, Polymeropoulos, & Wolfgang, 2008; Voineskos et al., 2011; Xu et al., 2010). However, their impact on prognosis of TBI is still poorly investigated.

2.4.3.1 Brain-derived neurotrophic factor

Brain-derived neurotrophic factor (BDNF) is the most abundant neurotrophin in the brain (Barde, Edgar, & Thoenen, 1982; Leibrock et al., 1989). It plays an important role in the survival, differentiation, synaptic plasticity and outgrowth of peripheral and central neurons throughout adulthood (Huang & Reichardt, 2001; Poo, 2001).

The rs6265 is an A to G polymorphism of *BDNF* gene. The more common G allele encodes for valine, while the A allele encodes for methionine (Leibrock et al., 1989). The SNP in the heterozygous form produces a mature protein that has altered intrinsic biological activity. This alteration affects intracellular processing and secretion of mature BDNF. Therefore, neuroplastic effect of BDNF in the heterozygous form is compromised, albeit in an unclear fashion, compared to the homozygous BDNF (Egan et al., 2003). BDNF is found abundantly in the hippocampus, an area which plays an important role in memory, attention, and speed of information processing (Farkas & Povlishock, 2007).

2.4.3.2 Glial-derived neurotrophic factor

Glial-derived neurotrophic factor (GDNF) promote the survival and differentiation of dopaminergic neurons, protects dopamine neurons from some neurotoxins and enhances high-affinity uptake of dopamine (Lin, Doherty, Lile, Bektesh, & Collins, 1993; Xiao, Hirata, Isobe, & Kiuchi, 2002).

The rs36119840 is an A to G polymorphism of the *GDNF* gene. GDNF is a glycosylated, disulfide-bonded, homodimer that is a distantly related member of the transforming growth factor-beta superfamily. In embryonic midbrain cultures, recombinant human GDNF promoted the survival and morphological differentiation of dopaminergic neurons and increased their high-affinity dopamine uptake (Lin et al., 1993). However, the role and function of this polymorphism and how it affects the mature protein is unknown.

2.4.3.3 Ciliary neurotrophic factor

Ciliary neurotrophic factor (CNTF), which is expressed mainly by astrocytes is a member of the interleukin-6 family of cytokines that regulates neuro-inflammatory responses. CNTF was identified as a survival and differentiation factor for a variety of neuronal cell types, including motor, sensory and sympathetic neurons (Barres, Schmid, Sendnter, & Raff, 1993; Louis, Magal, Takayama, & Varon, 1993; Sendtner, Carroll, Holtmann, Hughes, & Thoenen, 1994).

The rs1800169 is a null mutation of the *CNTF* gene. The G to A transition produces a new splice acceptor site and the resulting mRNA codes for an aberrant protein (Barres et al., 1993; Louis et al., 1993; Sendtner et al., 1994; R. Takahashi et al., 1994). CNTF supports survival and/or differentiation of a variety of neuronal cell types including sensory, sympathetic, and motoneurons. Furthermore, nonneuronal cells, such as skeletal muscle cells, microglial cells, oligodendrocytes and liver cells, respond to exogenously administered CNTF, both in vitro and in vivo (Sendtner et al., 1994).

2.5 Serum Protein biomarkers

Biomarkers arising from brain tissue after TBI will eventually find their way into the cerebrospinal fluid, and subsequently into the circulating blood, although in much lower levels. Since collection of peripheral blood samples is much easier than collection of cerebrospinal fluid in clinical practice, many candidate biomarkers of TBI of interest have been assessed in serum. These biomarkers could indirectly reflect on the status of the brain (Wang et al., 2005). To date, research data on the potential usefulness of various serum biomarkers such as S100B protein, Glial Fibrillary Acidic Protein, Neuron Specific Enolase, Myelin Basic Protein, Fatty Acid-binding Proteins, Creatine Kinase Brain Isoenzyme, Inflammatory Markers, Oxidative Stress Markers, Markers of Vascular Injury, Heat Shock Proteins, and Neuroendocrine Markers are available.

2.5.1 S100B protein

The S100B is a 10.5 kDa, calcium binding protein which is synthesized and secreted by astrocytes. It is one of the most comprehensively studied biomarker of TBI. Serum S100B has been shown to be a sensitive marker for brain injury, which correlates with the severity of the injury (Herrmann et al., 2000; Savola et al., 2004). Elevated S100B is associated with secondary complications but only after complications have set in, not before. Hence, daily S100B levels cannot predict the occurrence of secondary complications, thus limiting its usefulness (Unden et al., 2007). A significant correlation was also demonstrated between the volume of contusion visible on computed tomography (CT) scan and increased serum S100B level (Herrmann et al., 2000; Raabe et al., 1998). In studies by Raabe et al. (1998) and Vos et al. (2004), S100B strongly predicted a poor outcome in patients with severe TBI (Raabe et al., 1998; Vos et al., 2004); and serum S100B levels at 24-hour post injury could also serve as a screening tool for the early

detection of patients at risk for brain death after severe TBI (Egea-Guerrero et al., 2013). It was also reported that serum S100B was increased in pediatric TBI (Berger et al., 2005). A higher S100B concentration was also associated with severe pediatric TBI with the lowest GCS score on admission (Piazza et al., 2007), and with a worse outcome (Berger, Beers, Richichi, Wiesman, & Adelson, 2007). However, early increase of this protein is not a reliable prognostic index of neurological outcome, since even very elevated values are compatible with complete neurological recovery (Piazza et al., 2007). Meanwhile, several studies have shown that S100B is not a TBI specific protein, having been reported to be elevated after bone fractures, thoracic contusions without fractures, burns and even after minor bruises (Anderson, Hansson, Nilsson, Dijlai-Merzoug, & Settergren, 2001). It is also not a specific marker of TBI if there is local ischemia and reperfusion of the kidney, liver and gut because local ischemia and reperfusion also cause an increase of S100B levels (Pelinka, Harada, et al., 2004). In the setting of polytrauma, interpretation is hard.

2.5.2 Glial fibrillary acidic protein

"Glial fibrillary acidic protein" (GFAP) is a brain-specific, monomeric intermediate filament protein expressed by astrocytes and comprises a major portion of the cytoskeleton. Missler et al. (1999) first documented that GFAP is released into the circulation very soon after TBI. They speculate that this provides an early indication of the volume of brain parenchyma involved in TBI (Missler, Wiesmann, Wittmann, Magerkurth, & Hagenstrom, 1999). Serum GFAP levels correlated significantly with the injury severity score and CT scan findings. In patients with lower GCS and larger contusions (Vos et al., 2004) and in patients with intracranial pressure of 25 mm Hg or more (Pelinka, Kroepfl, et al., 2004), serum GFAP levels were elevated. Serum GFAP

level reaches a peak on the first day of TBI and later drops gradually (Nylen et al., 2006). It is not released in the setting of polytrauma without TBI (Pelinka et al., 2004). Higher levels of serum GFAP were measured in patients who died (Pelinka et al., 2004; Vos et al., 2004) or had a poor outcome at 6 months (Vos et al., 2004) or 1 year (Nylen et al., 2006) post injury compared to those who were alive or had good outcomes.

2.5.3 Neuron Specific Enolase

"Neuron Specific Enolase" (NSE) is one of the five isozymes of a glycolytic enzyme. It was originally found to be expressed in neurons and later also identified in oligodendrocytes, neuroendocrine cells, erythrocytes and thrombocytes. Serum NSE levels were significantly elevated in patients with major head injury (Ross, Cunningham, Johnston, & Rowlands, 1996). NSE levels also correlated significantly with the injury severity score and CT scan findings (Skogseid, Nordby, Urdal, Paus, & Lilleaas, 1992; Vos et al., 2004). Patients with moderately severe to severe TBI (GCS score at the site of accident < or =12) (Herrmann et al., 2000) and those patients with larger contusions had higher serum NSE levels (Herrmann et al., 2000; Skogseid et al., 1992). In pediatric TBI, elevated serum NSE significantly predicts for intracranial lesions in children with blunt head trauma but it is neither sensitive nor specific enough to predict intracranial lesions in all patients (Fridriksson, Kini, Walsh-Kelly, & Hennes, 2000). Patients who died or had a poor outcome 6 months post injury had significantly higher NSE levels than those who were alive or had good outcome (Herrmann et al., 2001; Vos et al., 2004). However, recent findings showed that serum NSE level may also be elevated in non-traumatic conditions such as neuroendocrine bladder tumors, small cell lung cancer, neuroblastoma and stroke (Schoerkhuber et al., 1999).

2.5.4 Myelin Basic Protein

"Myelin Basic Protein" (MBP) is a main and specific protein component of myelin. It has a molecular weight of 18.5 kDa. It was reported that serum MBP is increased in pediatric TBI and raised MBP level is mainly correlated with a worse outcome in children (Berger et al., 2005). However, it may also be released into the circulation in demyelinating disease (Ingebrigtsen & Romner, 2002).

2.5.5 Fatty acid-binding proteins

There are two types of "fatty acid-binding proteins" (FABP) expressed in various tissues, the "brain type" (B-FABP) and the "heart type" (H-FABP). B-FABP is found solely in the brain. Pelsers et al. (2004) showed that the highest concentrations of B-FABP and H-FABP is in the frontal lobe and pons, with the concentration of H-FABP at least 10 times higher than B-FABP. B-FABP was not detected in serum from healthy donors whereas, the level of serum H-FABP was 6 mg/L. In the same study, they reported that serum B-FABP was increased in 68% and H-FABP was above its upper reference limit in 70% of the patients with mild TBI (n= 130). B-FABP and H-FABP were found to be more sensitive markers for mild TBI than S100B and NSE (Pelsers et al., 2004). In a recent study, serum H-FABP levels at 48 hour were inversely correlated with the outcome at 3 months and were able to predict mortality with 75% sensitivity and 93% specificity (Walder et al., 2013).

2.5.6 Creatine Kinase Brain Isoenzyme

Creatine kinase brain isoenzyme (CKBB), synthesized by astrocytes, is an isoenzyme of creatine kinase. It has a molecular mass of 40 to 53 kDa. CKBB is released when there is anatomical injury to the brain. With a short half-life it is rapidly eliminated from circulation, hence it increases during the first few hours of trauma and rapidly drops unless there is continuous release. Furthermore, to be released into the circulation, blood brain barrier disruption is necessary (Schwartz, Bazan, Gage, Prihoda, & Gillham, 1989). In a study of 60 patients, Skogseid et al. (1992) found increased serum CKBB in 88% of the patients (n = 18) with moderate to severe head injury and in 23% of patients (n = 42) with minor head injury. They concluded that concentrations of CKBB correlated with the severity of injury as assessed clinically, and with the volume of contusion as estimated from CT scans (Skogseid et al., 1992). However, CKBB is also elevated in adenocarcinomas involving the prostate, ovary, gastrointestinal tract and breast, and small cell anaplastic carcinoma of the lung making CKBB not that specific for TBI (Schwartz et al., 1989).

2.5.7 Inflammatory Markers

Increased serum concentrations of “pro-inflammatory cytokines” and “anti-inflammatory cytokines”, “chemokines”, and “acute phase reactant proteins” have been observed as potential markers for TBI. Pro-inflammatory cytokines (IL-1, TNF-α, IL-6), anti-inflammatory cytokines (IL-10, TGF-β), chemokines (ICAM-1, macrophage inflammatory protein [MIP-1, MIP-2], acute phase proteins (amyloid A, C-reactive protein) have been reported to change in various bodily fluids in response to TBI.

The candidate of much interest, IL-1β, is elevated in serum in both children and adults with TBI (Chiaretti et al., 2005; Singhal et al., 2002). The increase in IL-1β expression was correlated with head injury severity, and was indicative of poor clinical outcome in children (Chiaretti et al., 2005). These studies also showed that elevated IL-6 was also associated with head injury severity in both children and adults (Chiaretti et al., 2005; Minambres et al., 2003) and unfavourable outcome in children (Chiaretti et al., 2005). Serum TNF-α was also upregulated after TBI and aids in stimulating neutrophil and recruit monocyte to the injury site to phagocytose cellular debris (Crespo et al., 2007; Goodman, Robertson, Grossman, & Narayan, 1990; S. A. Ross, Halliday, Campbell, Byrnes, & Rowlands, 1994). In severe TBI, it was found that TNF-α upregulation correlated with severity, but did not correlate with fatal outcome (Crespo et al., 2007). Sohrevardi et al. (2013) reported that on the 7th day of admission, serum IL-8 levels in patients with diffuse axonal injury had a negative correlation with GCS and GOS (Sohrevardi et al., 2013).

Csuka et al. (1999) also reported that serum IL-10 were increased after severe TBI showing a peak during the first and second days followed by a lower rise in the second week (Csuka et al., 1999). IL-10 elevation in serum is a candidate marker to predict which patient will develop intracranial hypertension and cerebral hypoperfusion (Stein et al., 2012). Although TGF-β is another candidate of much in interest in TBI, it could not be correlated with the extent of initial injury by CT scan or by GOS (Morganti-Kossmann et al., 1999). However, in a cohort of TBI patients who had diffuse axonal injury, the level of TGF-β was found to be increasing after admission and reached a maximum level on the 7th day (Sohrevardi et al., 2013).

Hergenroeder et al. (2008) reported that C-reactive protein is rapidly elevated after brain trauma and showed that it was a robust indicator of injury even at a very early timepoint of injury. However, C-reactive protein is also elevated when there is infection or other extracranial trauma (Hergenroeder et al., 2008).

Since injury to other organs can also increase the serum level of inflammatory markers in the patient's serum, these markers by themselves do not offer a high specificity for TBI. However, in certain circumstances in which selective TBI is suspected as in abusive TBI due to the shaken baby syndrome, these markers can be helpful to support a diagnosis of TBI (Berger et al., 2006).

2.5.8 Oxidative Stress Markers

The brain has a high oxygen demand and is very vulnerable to oxidative stress. During normal aerobic metabolism, superoxide radicals are constantly produced and scavenged by a number of antioxidant enzymes, including superoxide dismutase, glutathione reductase and catalase. Additionally, chemical antioxidants such as glutathione, ascorbic acid, and vitamin E are also likely to be involved in the detoxification of free radicals. Hypoperfusion and cell ischemia associated with TBI leads to oxidative stress arising from an increase in superoxide radicals. Superoxide radicals cause protein oxidation, lipid peroxidation and DNA damage (Griesbach, Hovda, Gomez-Pinilla, & Sutton, 2008). Lipid peroxidation of neuronal membranes which have an abundance of polyunsaturated fatty acids (Won, Kim, & Gwag, 2002), causes membrane dysfunction and cell death by lysis (Zink, 2001). Superoxide radicals can also injure endothelial cells, contributing to vasogenic and cytotoxic oedema (Finfer & Cohen, 2001). When the brain is exposed to oxidative stress, activity and expression of antioxidant enzymes such as superoxide dismutase and catalase were shown to be

increased in animal models (Kucur et al., 2005). This has yet to be confirmed in human TBI cases.

2.5.9 Markers of Vascular Injury

The Von Willebrand Factor (VWF) is an adhesive glycoprotein that by interacting with components of the extracellular matrix and platelet receptors, stimulates onset and progression of thrombus formation at the site of vascular damage (Mendolicchio & Ruggeri, 2005). VWF is an established marker of endothelial activation, a pro-inflammatory and pro-coagulant condition of injured endothelial cells lining blood vessels. Yokota et al. (2002) reported that elevated serum VWF suggests endothelial activation in severe head injury. They demonstrated that serum VWF in focal brain injury was significantly higher than in diffuse brain injury. In addition, the serum level of VWF may also be able to predict patients who develop delayed traumatic intracerebral hematoma (Yokota et al., 2002). This is supported by another study which showed a positive correlation between VWF levels with scores in the Marshall CT classification. The Marshall CT classification in this study groups TBI patients into four ordinal categories, based on the status of the mesencephalic cisterns, the degree of midline shift in millimeters, and the presence or absence of one or more surgical masses on the CT scan. In this study, it was also suggested that an increase in VWF level following severe TBI may be a marker of unfavorable outcome, predicting mortality with a specificity of 68% (De Oliveira et al., 2007). Another study in children with severe cranio-cerebral trauma also demonstrated significantly increased VWF (Becker et al., 1999).

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptides that are vital for the degradation of extracellular matrix components such as collagen, fibronectins and elastins. During normal cell development activated metalloproteinases are needed for the extracellular matrix degradation to permit cell migration (Vu & Werb, 2000). Suehiro et al., studied patients with acute TBI and reported high serum levels of MMP-9. High levels of MMP-9 correlated with high levels of IL-6, suggesting that MMP-9 may be associated with post-TBI inflammatory events (Suehiro et al., 2004).

Maier et al. (2007) showed an increase of the serum levels of Endothelin-1, a very potent vasoconstrictor peptide, in patients with severe TBI at the acute stage. They also reported that it was much more prominent among patients with subarachnoid hemorrhage (Maier, Lehnert, Laurer, & Marzi, 2007).

2.5.10 Heat Shock Proteins

Heat shock proteins (HSP) are chaperone molecules that protect cells from external stress. They play an important role in the protein folding and unfolding as well as in the assembly and disassembly of protein complexes. The Hsp70kDa family (HSPs are designated according to their molecular weights) regulates cerebral processes in normal or stress conditions. Pittet et al. (2002) showed that the Hsp72 (a member of the Hsp70 family) can be detected in the serum of patients with severe trauma within 30 minutes after trauma and that high levels are associated with survival after severe trauma (Pittet et al., 2002). Another study reported that high serum Hsp70 may also be a promising outcome biomarker in severe TBI as male patients with fatal outcome had higher serum concentrations compared with survivors (da Rocha et al., 2005).

2.5.11 Neuroendocrine Markers

Endocrine function may be affected by various inflammatory mediators, including cytokines and free radicals (Tanriverdi et al., 2006), part of the acute adaptive response to trauma. Medications such as glucocorticoids, narcotics or dopaminergic agents that may be administered at this stage may also influence endocrine function. Gonadotropin and somatotropin deficiency, followed by corticotropin and thyrotropin deficiency, and hyper- or hypoprolactinaemia are the most common alterations (Bondanelli, Ambrosio, Zatelli, De Marinis, & degli Uberti, 2005). Schneider et al., combined data from 19 studies, which includes 1137 patients, and showed that the prevalence of hypopituitarism was greater in patients with severe compared to mild or moderate TBI (Schneider, Kreitschmann-Andermahr, Ghigo, Stalla, & Agha, 2007). Tanreverdi et al., studied 52 TBI patients (43 men and 9 women) and reported that 5.8% had thyroid stimulating hormone deficiency, 41.6% gonadotropin deficiency, 9.8% adrenocorticotropic hormone deficiency, and 20.4% had growth hormone deficiency (Tanriverdi et al., 2006). Other studies reported cortisol hyporesponsiveness and hypogonadism as the most common deficiencies at the acute stage of TBI (Agha et al., 2004; Dimopoulou et al., 2004). Aldosterone levels were found to be high in patients with severe cerebral contusion, in patients whose CT scans showed a marked midline shift and disappearance of the perimesencephalic cistern, and in epidural hematoma. Moderately increased levels were found in patients with a slight midline shift (Takahashi, Sato, & Tsuji, 1989).

CHAPTER 3

APOE GENE AND ITS PROMOTER: INFLUENCES ON 6 MONTH OUTCOME AFTER TRAUMATIC BRAIN INJURY

3.1 Introduction

Apolipoprotein E (ApoE) is a polymorphic protein with three common isoforms (ApoE2, ApoE3, and ApoE4), which are encoded by three alleles (ε2, ε3 and ε4) of the Apolipoprotein E gene (*APOE*), respectively. These three alleles differ by single nucleotide changes at positions 112 and 158. *APOE* ε2 has TGC at both positions, *APOE* ε3 has TGC at position 112 and CGC at position 158, whereas *APOE* ε4 has CGC at both positions. ApoE is a major CNS lipoprotein synthesized by astrocytes (Mahley, 1988; Mahley, Innerarity, Rall, & Weisgraber, 1984) and has been suggested to play a role in neuronal homeostasis (Laws, Hone, Gandy, & Martins, 2003), particularly, mobilization of cholesterol into the CNS where it is required for neuronal plasticity (Moestrup, Gliemann, & Pallesen, 1992; Rebeck, Reiter, Strickland, & Hyman, 1993). ApoE is also postulated to be involved with neuronal repair by mediating the recycle of damaged cell membranes (Laws et al., 2003). The association between polymorphisms of *APOE* gene and its promoter with outcome is still not fully understood, particularly in Asian populations. Wide inter-ethnic variations in the APOE gene and its promoter were thought to be responsible for these inconsistent findings. In Malaysia, there are 3 major ethnic groups: Malay, Chinese and Indian. These ethnicities, especially the latter two, have migrated to Malaysia from China and India in the last century. In this study we attempted to determine if polymorphisms in the *APOE* gene and its promoter influence the outcome in a large cohort of multiethnic Malaysian patients with TBI.

3.2 Literature Review

In a prospective evaluation of 89 patients with TBI, there was a 2-fold increase in unfavorable outcome at 6 months in patients with *APOE* ε4 compared to patients without *APOE* ε4. "Death", "vegetative state", or "severe disability" were defined as unfavorable outcomes using the GOS (G. M. Teasdale et al., 1997). Several other clinical studies concerning the severities and outcome of TBI in adults support the notion that *APOE* ε4 allele is associated with a poor prognosis and unfavorable outcome (Chiang et al., 2003; Friedman et al., 1999; Liaquat et al., 2002). Nonetheless, some contradicting evidence suggest that *APOE* ε4 has no effect on recovery from TBI (Alexander et al., 2007; Chamelian et al., 2004; Diaz-Arrastia et al., 2003; Millar et al., 2003; Nathoo et al., 2003; Pruthi et al., 2010; G. M. Teasdale et al., 2005; Willemse-van Son et al., 2008). However, most of these reports document the effect of *APOE* ε4 on TBI outcome almost exclusively in white populations or those derived from Caucasians. Among a black population in South Africa with a relative high frequency of *APOE* ε4 allele, *APOE* ε4 allele was not found to have a significant effect on TBI outcome (Nathoo et al., 2003). Other studies showed that *APOE* genotype has little or insignificant effect on outcome in relatively mild injuries (Chamelian et al., 2004a; Sundstrom et al., 2004). Therefore, more research into the association between *APOE* polymorphism and outcome after TBI is needed, particularly in non-Caucasian racial groups.

Recently, three upstream SNPs in the *APOE* gene promoter at positions -491 A/T, -427 C/T, and -219 G/T (also known as Th1/E47cs) have been reported. The various allelic combinations at each position viz., -491 AA or -491 AT or -491 TT; -427 CC or -427 CT or -427 TT; -219 GG or -219 GT or -219 TT, could alter transcription of *APOE* gene (Artiga, Bullido, Sastre, et al., 1998). Poor recovery from TBI was reported to be more frequent among subjects with the TT genotype of -219 G/T compared to the GG

and GT genotypes. However, no association was noted between unfavorable outcome and the -491 A/T promoter polymorphism (Lendon et al., 2003). To date, there is no study on -427 C/T promoter polymorphisms and outcome.

3.3 Method

3.3.1 Patient cohort

"A total of 205 unpreselected and consecutive TBI patients admitted to the Neuro Intensive Care Unit, University of Malaya Medical Centre, were prospectively recruited for this study between Feb 2009 and June 2012. Baseline data including, gender, age, cause of TBI and clinical severity as indicated by the GCS, were recorded on admission. Injuries were graded as mild (GCS 15–14), moderate (GCS 13–9), and severe (GCS 8–3) (Teasdale & Jennett, 1974, 1976). Patients who had blood transfusions, and who had co-morbidities such as polytrauma, hypertension, Alzheimer's disease and other major systemic diseases that might interfere with TBI related disability, were excluded from the study. The Ethics Committee of the University Malaya Medical Centre had approved the study protocol" (Anada, Ganesan, Ramahsamay, & Wong, 2012).

(Note: The incidents of TBI are high in Malaysia, however, these includes TBI accompanied with other major systemic injuries (Liew et al., 2009). The incidents of sole TBI incidents are relatively lower (300 cases per year) and a study of 200 subjects are sufficient for 80% sample power calculated using STATA statistical software.)

3.3.2 DNA extraction and purification

"Two mls of EDTA blood were collected from each patient after written consent from patient or patient's next of kin, and the blood samples were stored at -80°C until DNA extraction. Blood samples were washed in 1X standard saline citrate buffer at room temperature and digested for 1 hour using proteinase K and sodium-dodecyl-sulphate at 55°C. Digested products were purified using phenol-chloroform-isoamyl alcohol (25:24:1, pH 8.0, Sigma, USA) extraction method (Davis, Dibner, & Battey, 1986). The DNA was precipitated from the aqueous layer using 2M NaCI and cold absolute ethanol, air dried overnight, solubilised in 10:1 TE buffer and stored at -20°C for PCR. The purity and concentration of DNA were measured spectrophotometrically" (Anada et al., 2012). (Full details are found in Appendix B).

3.3.3 PCR and sequencing

"The *APOE* genotype and its promoter's SNPs were determined by PCR amplification. For the *APOE* gene a sense primer 5'-CTG GAG GAA CAA CTG ACC CCG GTG- 3' and anti- sense primer 5'-CAG GCG CTC GCG GAT GGC GCT GAG-3' that flank the positions 112 and 158 were used (Appendix C). For the promoter region, sense primer 5'-GGG GCT CCC CTG TGC TCA AG- 3' and anti-sense primer 5'- TGT TCT CCC CCT GCC CCA GG- 3' which flank positions -491, -427 and -219 were used (Appendix D). PCR was performed using Taq DNA Polymerase (Fermentas, Canada) with initial denaturation at 95ºC for 3 min, followed by 35 cycles of 60 sec of denaturation at 94ºC, 45 sec of annealing at 68ºC, and 30 sec of extension at 72ºC. After 10 min of final extension at 72ºC, the PCR products were kept in 4ºC. The PCR products were detected by electrophoresis in a 1.5% agarose gel, gel-purified as per manufacturer's instruction

(Qiagen, USA) and sent for direct PCR sequencing (First BASE Laboratories, Malaysia). The sequence results were analysed with the Sequence Scanner (Version: 1.0, Applied Biosystems, USA)" (Anada et al., 2012).

3.3.4 Outcome evaluation

Functional outcome was measured by GOS. The GOS categorizes functional outcomes into five levels: "1, death"; "2, persistent vegetative state"; "3, severe disability"; "4, moderate disability" and "5, good recovery" (Jennett & Bond, 1975). These data were collected at 6 month post-injury during a face-to-face interview whenever possible or by phone when direct interview was not possible using a standard evaluation form (Appendix G). Data on mortality was collected from patients' medical records. A GOS of 1, 2 and 3 was categorized as unfavorable outcome, whereas 4 and 5 was categorized as favorable outcome. GOS assessment was done without prior knowledge of patient's *APOE*/promoter or any other genotype results.

3.3.5 Statistical analysis

Analysis was performed using STATA, version 9.0. Independent T Test was used to compare variables within two groups. ANOVA was used to compare variables within three or more groups. Chi- square test, linear and logistic regression were used to determine association between variables.

3.4 Results

3.4.1 Demography and genotype frequencies

In this study of 205 cases of TBI (Table 3.1), the patients were predominantly male (86.8%), young (16-30 years old) and had moderate brain injury (GCS 9-13). Among our subjects, TBI was caused mainly by motor vehicle accidents, with motorcyclists recording the highest number of TBI cases (133/205 or 64.9%). This was followed by car drivers (6 subjects, 2.9%) and pedestrians (7 subjects, 3.4%). The second major cause of TBI was fall (25 subjects, 12.2%). Other causes of TBI were fall from a height (4 subjects, 2.0%) and assault (10 subjects, 4.9%). In this cohort, the cause of TBI for 16 subjects were unknown.

Table 3.1: Characteristics of 205 traumatic brain injury patients and *APOE* ε4 genotype.

***APOE* Genotype**	**No. of patients without ε4 (n= 149)**	**No. of patients with ε4 (n= 56)**	**Total**
Nature of accident			
MVA			
Motorcycle	94	39	133
Car	5	1	6
Other Vehicles	4	0	4
Pedestrian	6	1	7
Fall	17	8	25
Fall from a height	3	1	4
Assault	10	0	10
Unknown	10	6	16
Gender			
Male	131	47	178
Female	18	9	27
Age			
16-30	70	25	95
31-45	37	17	54
>45	42	14	56
Outcome			
Favorable (GOS 4-5)	123	26	149
Unfavorable (GOS 1-3)	26	30	56
GCS			
14-15	29	12	41
9-13	69	23	92
<9	51	21	72

Abbreviation: Motor Vehicle Accident (MVA), Glasgow Coma Score (GCS); Footnote: (1) For the subheading MVA, patient was using the vehicle when met with an accident; (2) A GOS of 1 (Death), 2 (Persistent Vegetative State) and 3 (Severe Disability) was categorized as unfavorable outcome

Each patient's *APOE* genotype and promoter SNP was determined by PCR amplification and direct sequencing. Figure 3.1 and Figure 3.2 show the typical electrophoresis gel results of *APOE* and its promoter's PCR products, respectively. These bands were excised, gel-purified and directly sequenced. The partial sequence electropherograms of *APOE* (Figure 3.1 to Figure 3.8) and its promoter region (figure 3.9 to Figure 3.11) are as shown. A nucleotide was identified as homozygous if it has a single peak and a relative peak height in a single channel that exceeded a threshold. A nucleotide was identified as heterozygous if it has two peaks and the signal was approximately half the expected homozygous peak height.

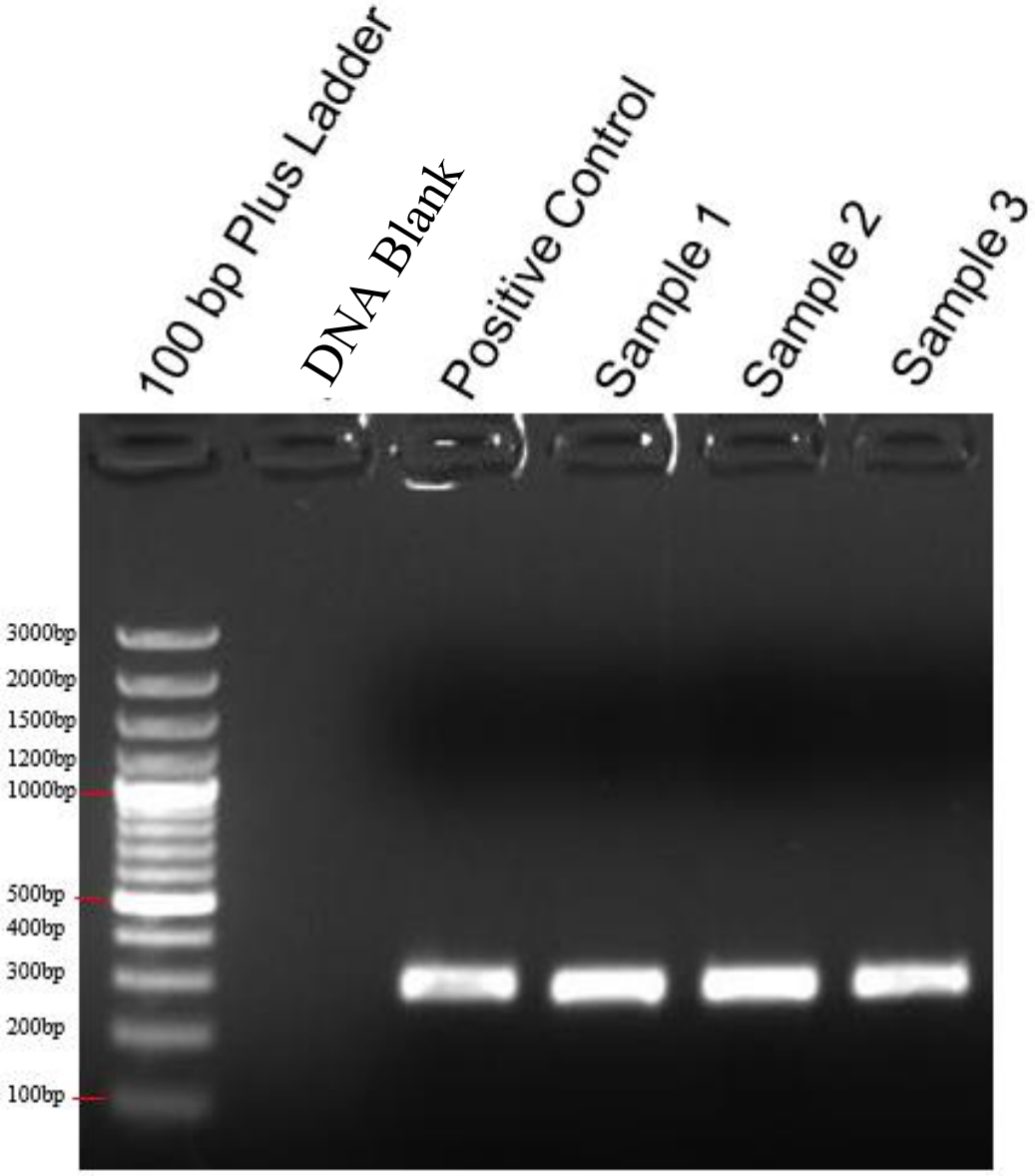

Figure 3.1: Electrophoresis gel of the *APOE* PCR product for 3 patient samples.

A sense primer 5'-CTG GAG GAA CAA CTG ACC CCG GTG- 3' and anti- sense primer 5'-CAG GCG CTC GCG GAT GGC GCT GAG-3' that flank the positions 112 and 158 of *APOE* gene produces a 320 base pair product. The DNA Blank PCR amplification was done by substituting DNA with ultrapure water. For the Positive Control, a previously sequence confirmed patient DNA was used. Sample 1-3 denotes PCR products for three different subjects.

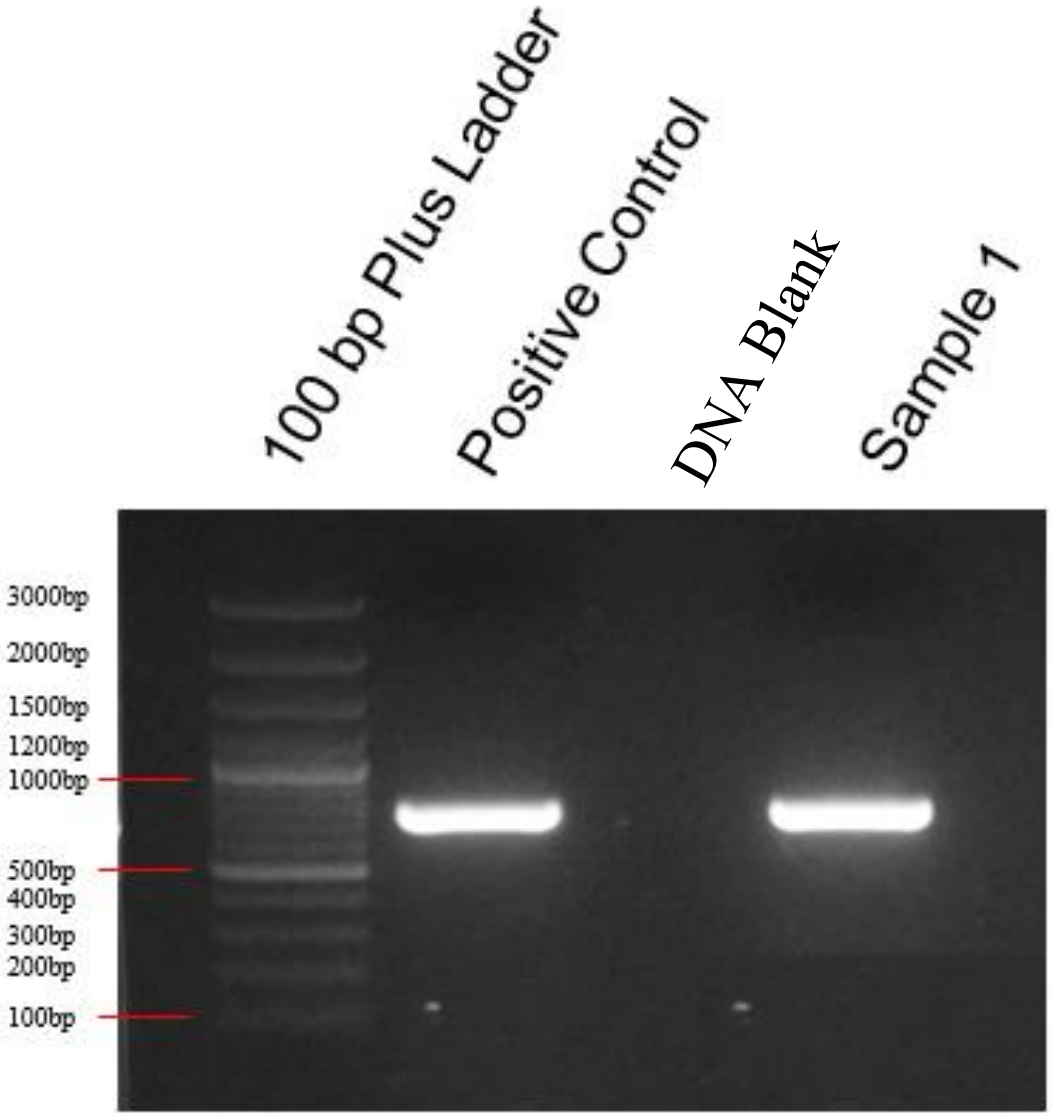

Figure 3.2: Electrophoresis gel of the *APOE* Promoter region PCR product for a patient.

A sense primer 5'-GGG GCT CCC CTG TGC TCA AG- 3' and anti-sense primer 5'-TGT TCT CCC CCT GCC CCA GG- 3' which flank positions -491, -427 and -219 of the *APOE* the promoter region produces a 680 base pair product. The DNA Blank PCR amplification was done by substituting DNA with ultrapure water. For the Positive Control, a previously sequence confirmed patient DNA was used. Sample 1 denotes PCR products for a subject.

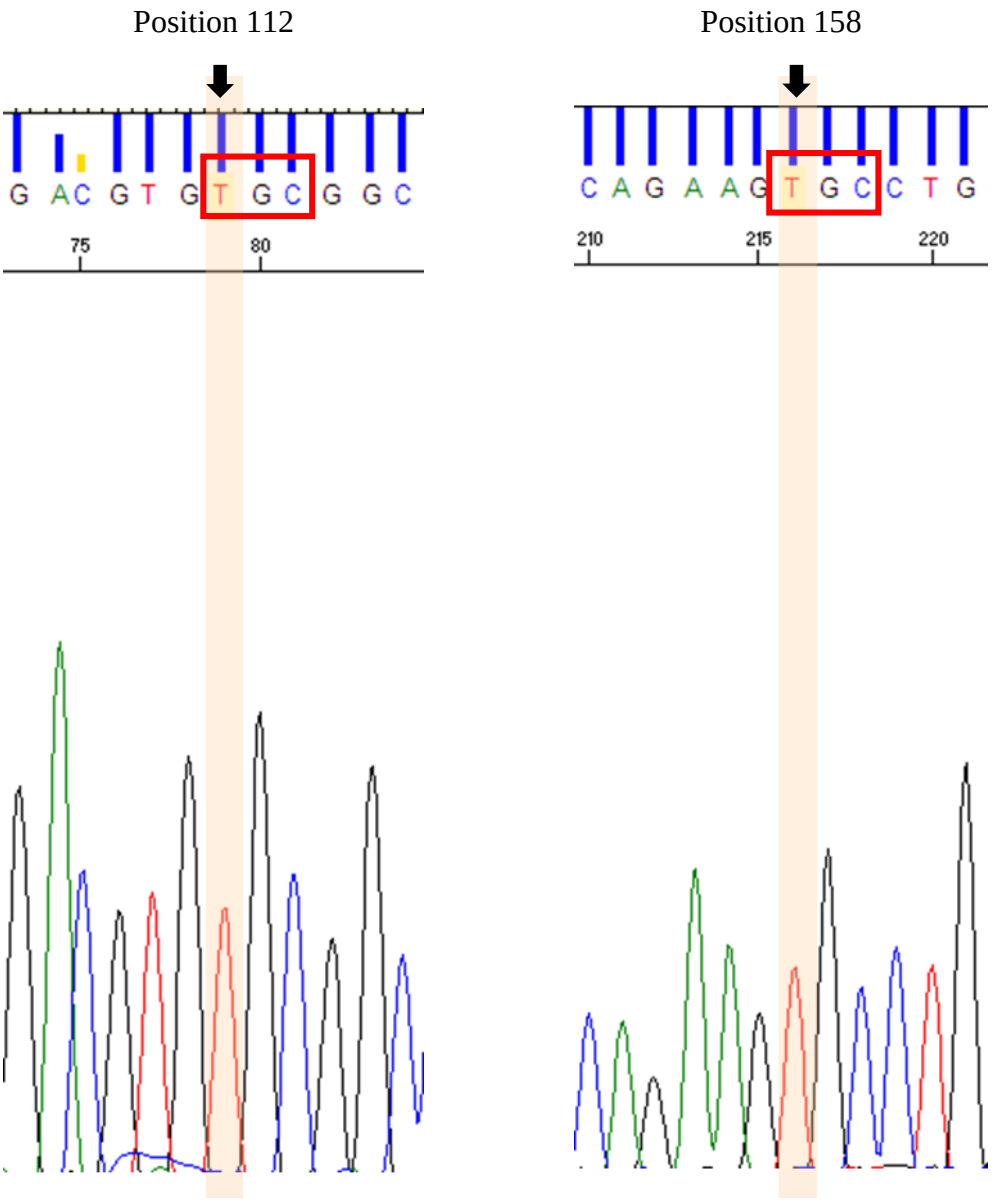

Figure 3.3: Partial sequence electropherograms of a subject with *APOE* ε2ε2 genotype.

A red peak indicates the presence of Thymine (T nucleotide) and blue peak indicates the presence of Cytosine (C nucleotide). The electropherogram showed a single red peaks at positions 112 and 158, thus the subject had homozygous T nucleotide at both positions 112 and 158, indicating the *APOE* ε2ε2 genotype.

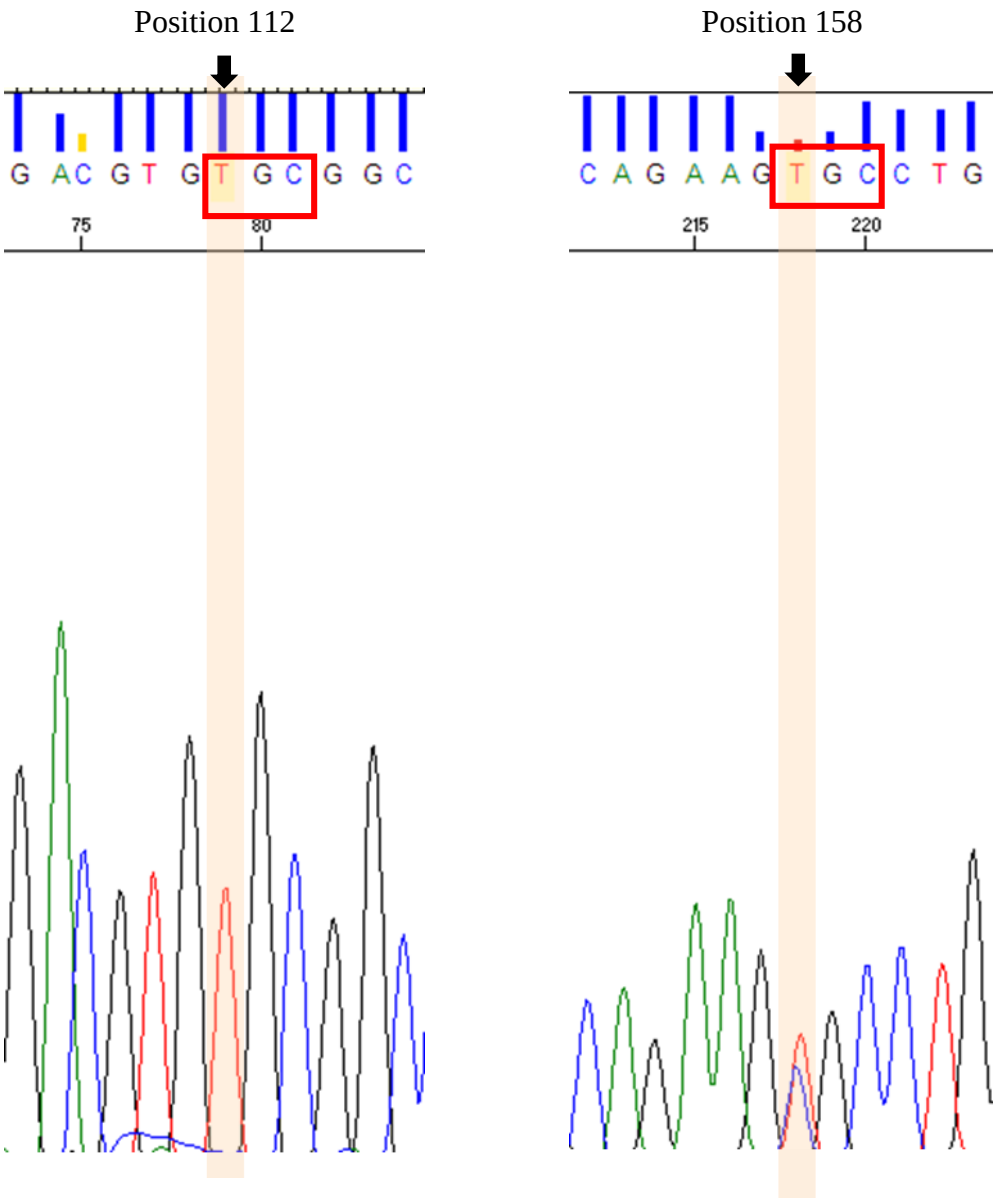

Figure 3.4: Partial sequence electropherograms of a subject with *APOE* ε2ε3 genotype.

A red peak indicates the presence of Thymine (T nucleotide) and blue peak indicates the presence of Cytosine (C nucleotide). The electropherogram showed a single red peak at position 112 and overlapping double peaks at position 158, thus the subject had homozygous T nucleotide at both positions 112 and heterozygous C/T at position 158, indicating the *APOE* ε2ε3 genotype.

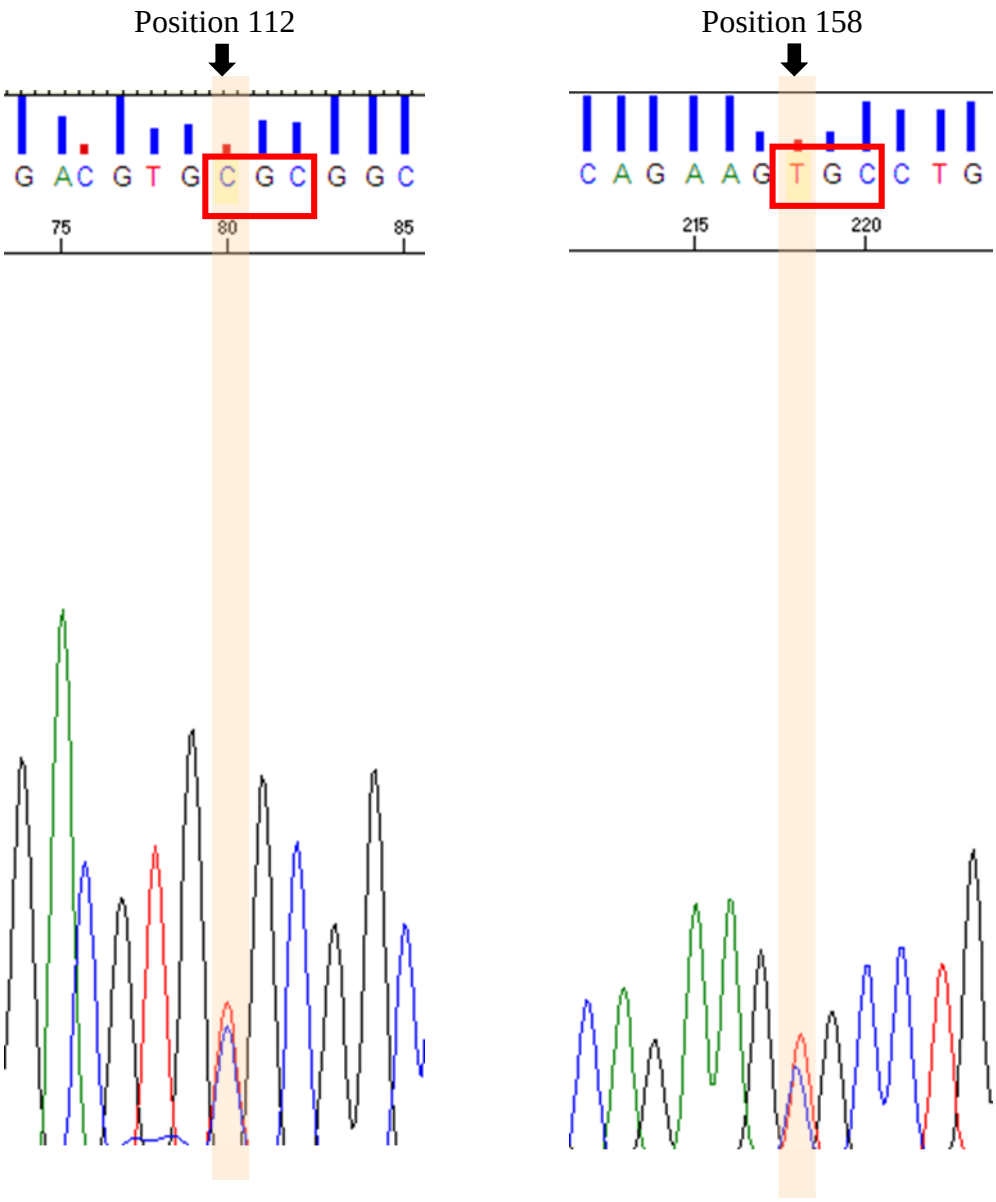

Figure 3.5: Partial sequence electropherograms of a subject with *APOE* ε2ε4 genotype.

A red peak indicates the presence of Thymine (T nucleotide) and blue peak indicates the presence of Cytosine (C nucleotide). The electropherogram showed overlapping double peaks at both positions 112 and 158, thus the subject had heterozygous C/T at both positions 112 and 158, indicating the *APOE* ε2ε4 genotype.

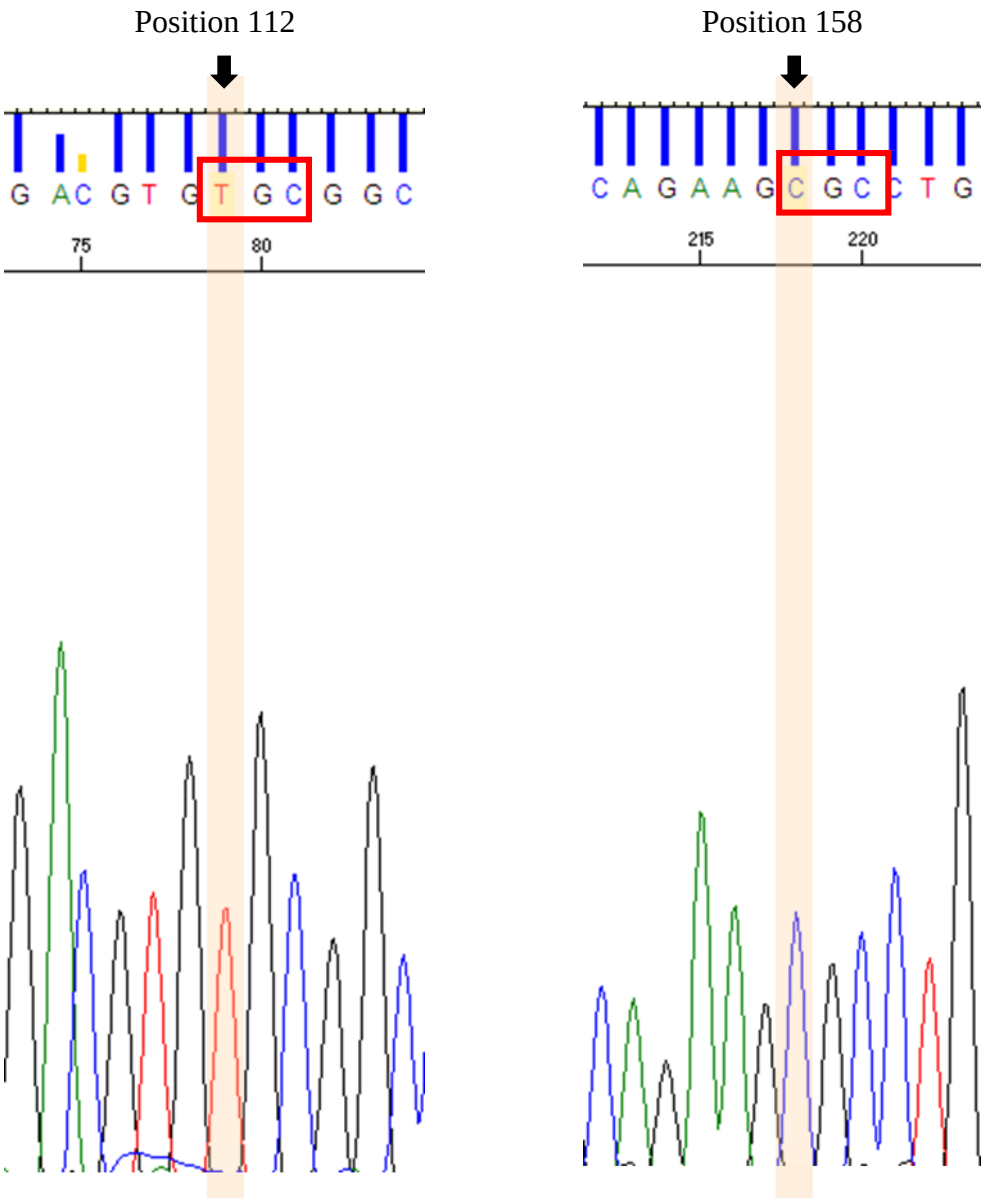

Figure 3.6: Partial sequence electropherograms of a subject with *APOE* ε3ε3 genotype.

A red peak indicates the presence of Thymine (T nucleotide) and blue peak indicates the presence of Cytosine (C nucleotide). The electropherogram showed a single red peak at positions 112 and a single blue peak at position 158, thus the subject have homozygous T nucleotide at position 112 and homozygous C nucleotide at position 158, indicating the *APOE* ε3ε3 genotype.

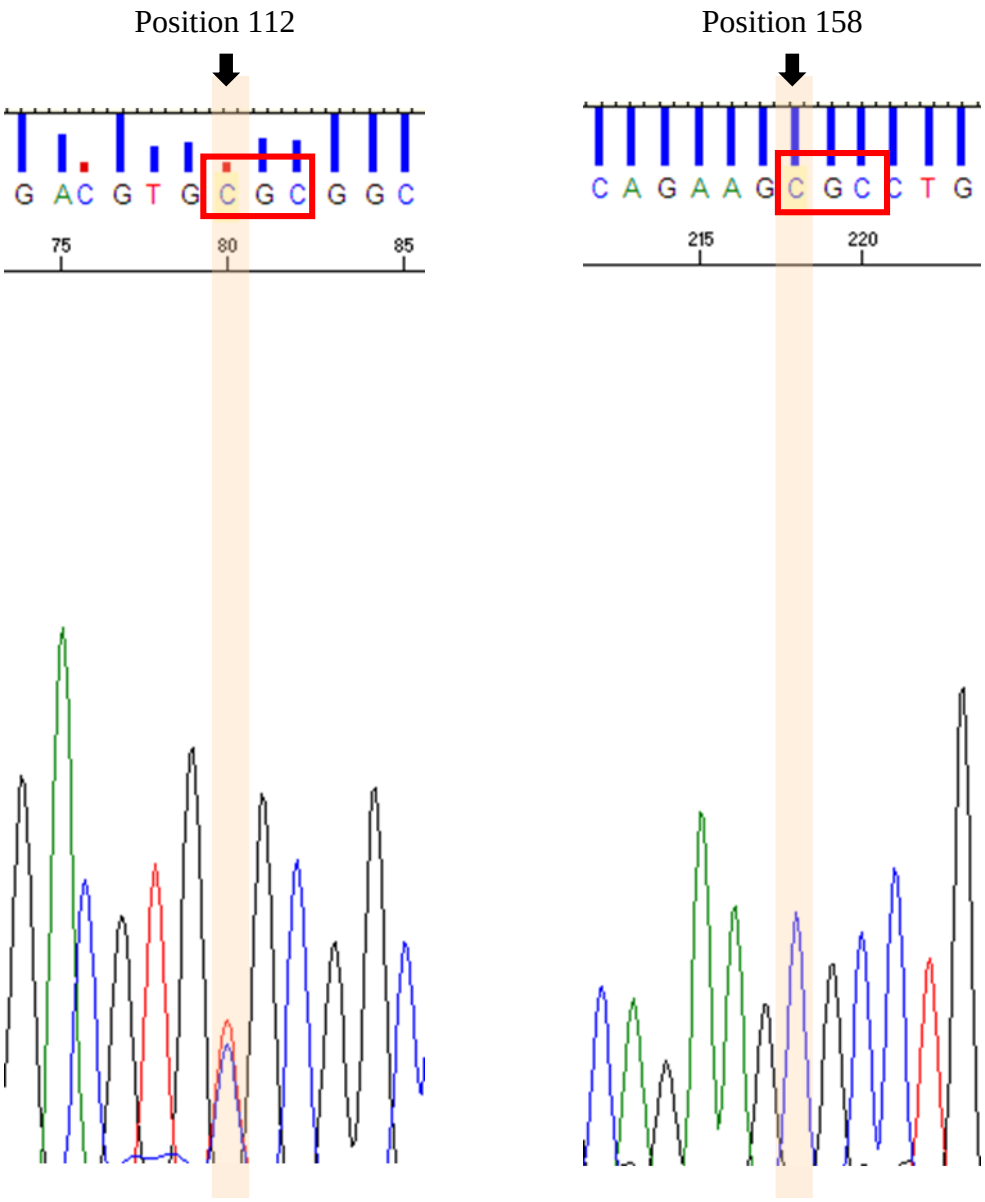

Figure 3.7: Partial sequence electropherograms of a subject with *APOE* ε3ε4 genotype.

A red peak indicates the presence of Thymine (T nucleotide) and blue peak indicates the presence of Cytosine (C nucleotide). The electropherogram showed overlapping double peaks at position 112 and a single blue peak at position 158, thus the subject have heterozygous C/T at position 112 and homozygous C nucleotide at position 158, indicating the *APOE* ε3ε4 genotype.

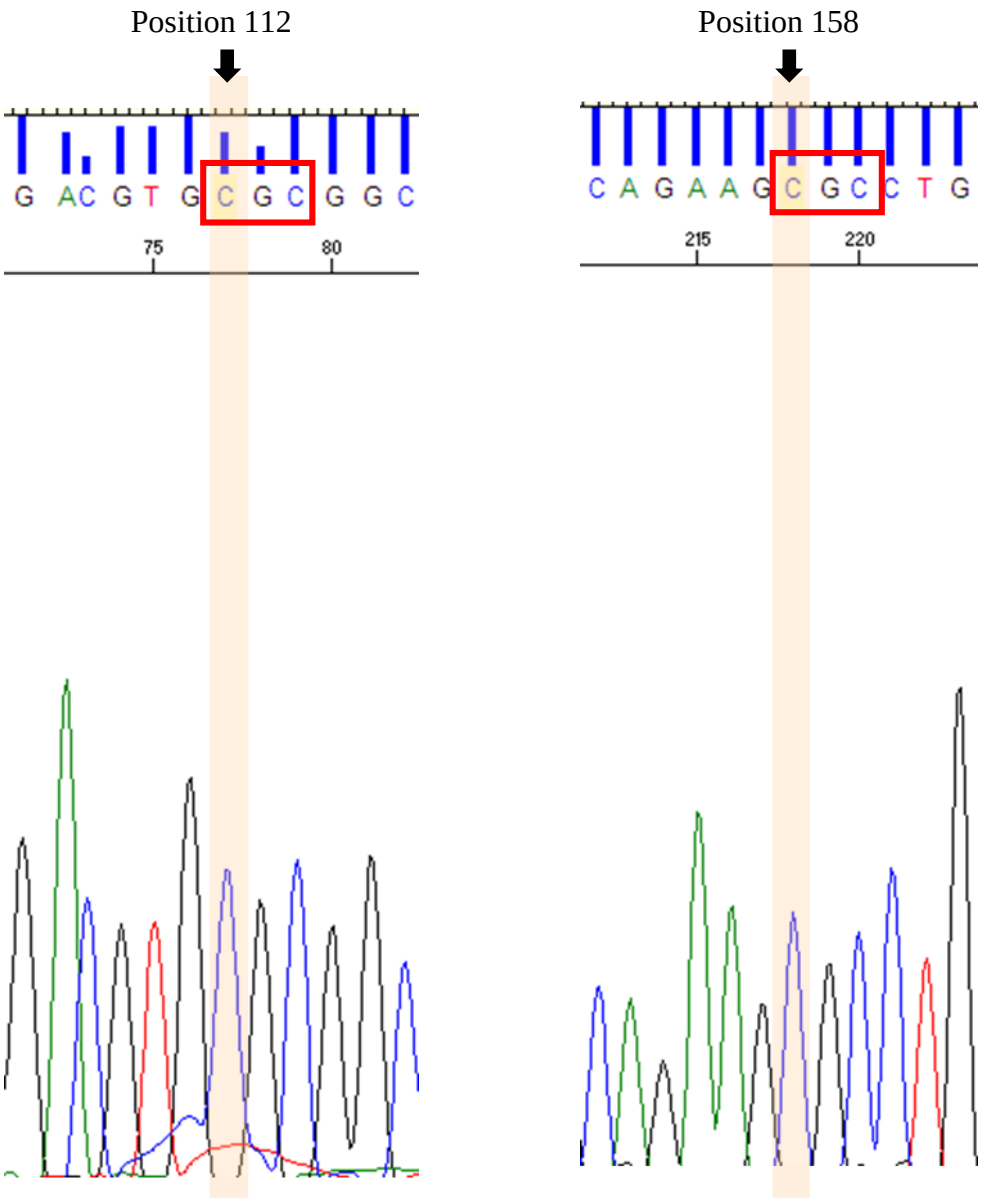

Figure 3.8: Partial sequence electropherograms of a subject with *APOE* ε4ε4 genotype.

A red peak indicates the presence of Thymine (T nucleotide) and blue peak indicates the presence of Cytosine (C nucleotide). The electropherogram showed a single blue peak at positions 112 and 158, thus the subject have homozygous C nucleotide at both positions 112 and 158, indicating the *APOE* ε4ε4 genotype.

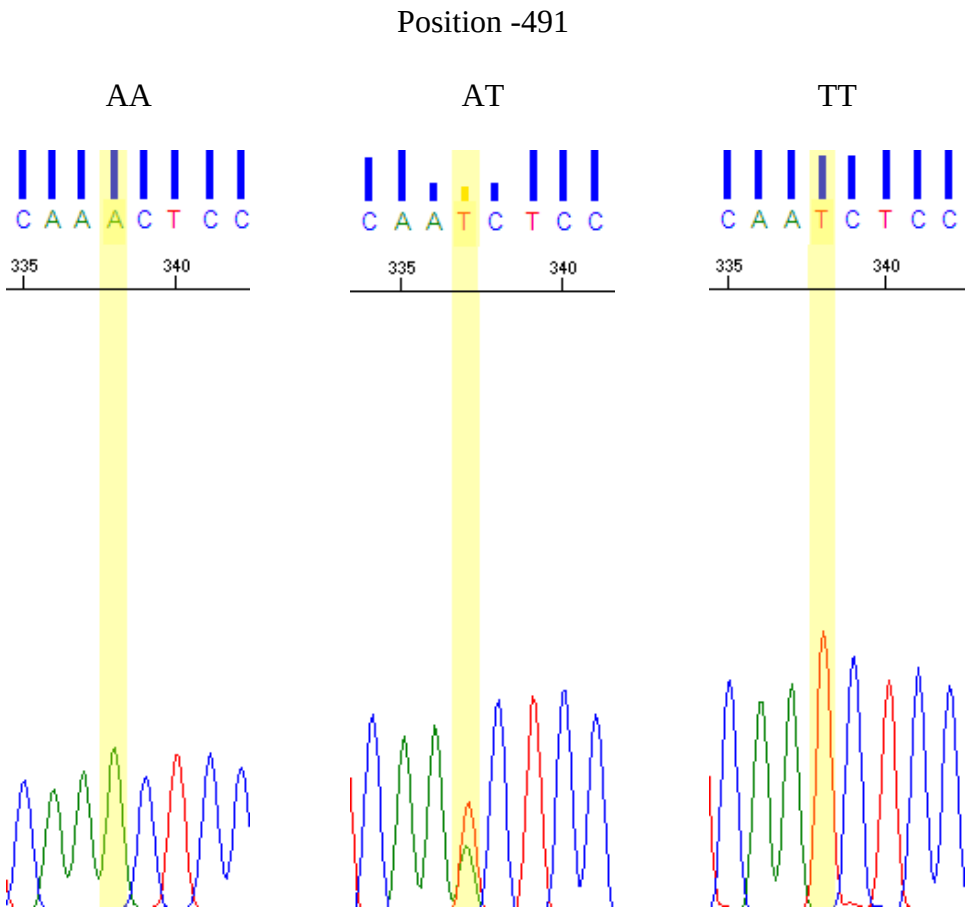

Figure 3.9: Partial sequence electropherograms of the *APOE* promoter region at position -491.

A red peak indicates thepresence of Thymine (T nucleotide) and a green peak indicates Adenine (A nucleotide). The electropherogram showed a single peak if the subject is homozygous at a polymorphism position (subject with homozygous T has single red peak and subject with homozygous A has a single green peak). The electropherogram showed two typical overlapping peaks if the subject is heterozygous AT.

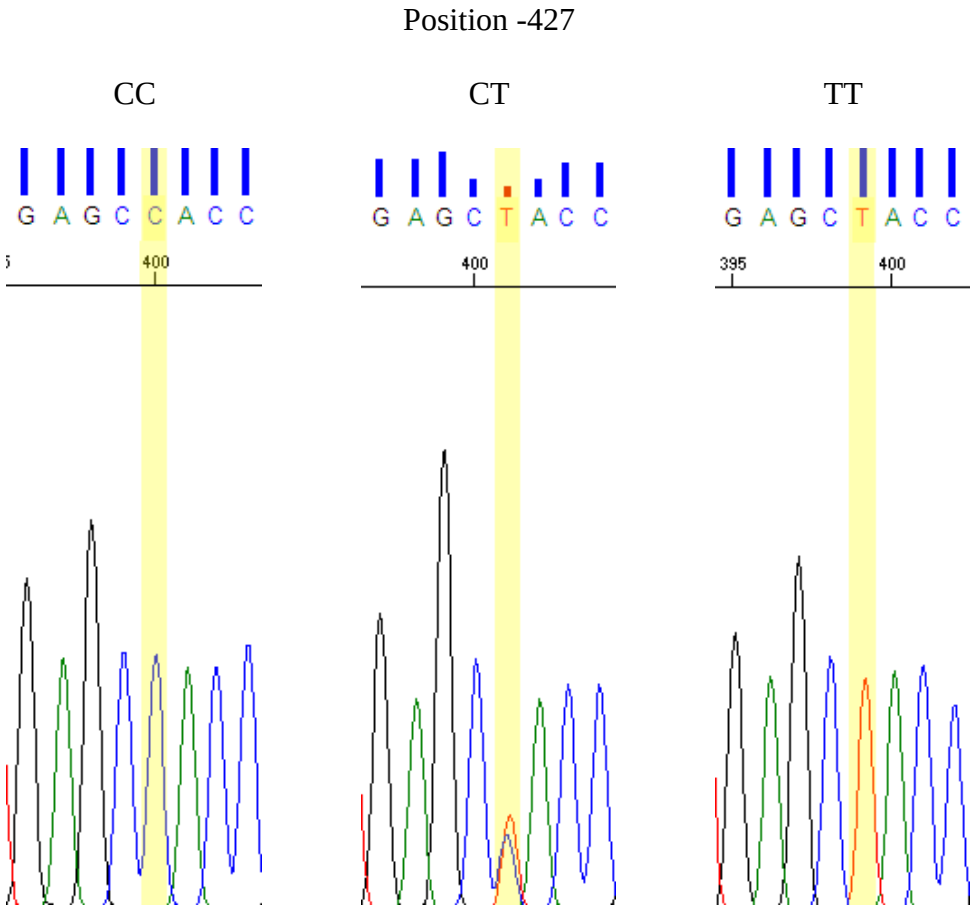

Figure 3.10: Partial sequence electropherograms of the *APOE* promoter region at position -427.

A red peak indicates the presence of Thymine (T nucleotide) and a blue peak indicate presence of Cytosine (C nucleotide). The electropherogram showed a single peak if the subject is homozygous at a polymorphism position (subject with homozygous T has a single red peak and subject with homozygous C has a single blue peak). The electropherogram showed two typical overlapping peaks if the subject is heterozygous CT.

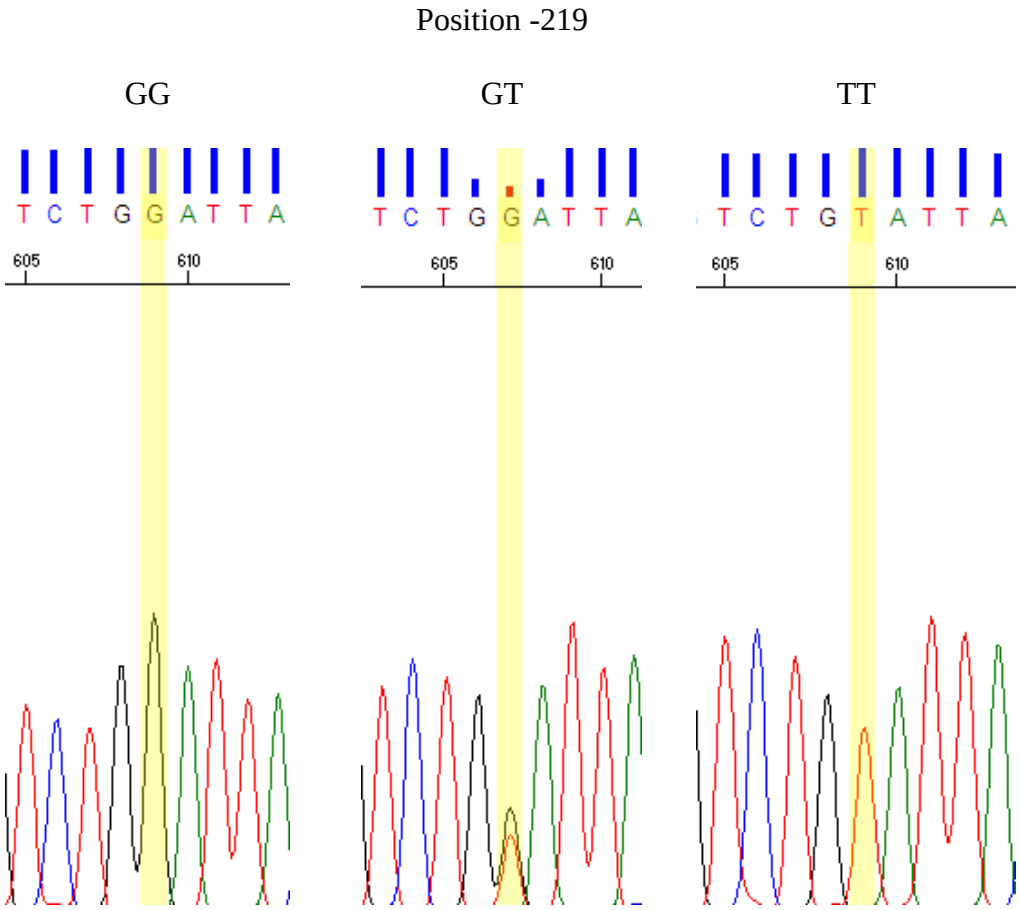

Figure 3.11: Partial sequence electropherograms of the *APOE* promoter region at position -219.

A red peak indicates the presence of Thymine (T nucleotide) and a black peak indicates presence of Guanine (G nucleotide). The electropherogram showed a single peak if the subject is homozygous at a polymorphism position (subject with homozygous T has a single red peak and subject with homozygous G has a single black peak). The electropherogram showed two typical overlapping peaks if the subject is heterozygous GT.

Fifty six patients (27.3%) had at least one copy of the *APOE* ε4 allele; the genotypes were: ε2ε4 (2.9%), ε3ε4 (21.5%) and ε4ε4 (2.9%). The other genotype frequencies were ε2ε2 (1.5%), ε2ε3 (10.7%), and ε3ε3 (60.5%) (Table 3.2).

Table 3.2: *APOE* genotype frequencies in 205 TBI patients

***APOE* Genotype**	**Frequency**	%
ε2ε2	3	1.5
ε2ε3	22	10.7
ε2ε4	6	2.9
ε3ε3	124	60.5
ε3ε4	44	21.5
ε4ε4	6	2.9

The frequencies of the polymorphisms found in the promoter region were: -491 AA (81.5%), -491 AT (17.5%), -491 TT (1.1%); -427 TT (80.5%), -427 CT (18.1%), -427 CC (1.7%) and -219 GT (47.8%), 219 TT (35.1%), -219 GG (17.3%) (Table 3.3).

Table 3.3: *APOE* promoter genotype frequencies in 205 TBI patients

***APOE* Promoter**		**Frequency**	%
-491 A/T			
	AA	167	81.5
	AT	36	17.5
	TT	2	1.1
-427 T/C			
	TT	165	80.5
	CT	37	18.1
	CC	3	1.7
-219 T/G			
	GG	35	17.3
	GT	98	47.8
	TT	72	35.1

3.4.2 Outcome after TBI

In our cohort of 205 patients (Table 3.4), 56 had unfavorable outcomes (GOS 1, 2, 3) after 6 months. Thirty of these patients had at least one copy of the *APOE* ε4 allele, whereas, in the 149 patients with favorable outcomes, only 26 had at least one *APOE* ε4 allele. Thus, overall, possession of an *APOE* ε4 allele appears to be significantly associated with an unfavorable outcome (OR: 5.5, 95% CI: 2.8-10.7, p= 0.01).

Table 3.4: Post-TBI outcome stratified by severity and presence of *APOE* ε4 allele

	No. of patients with favorable outcome (%)	**No. of patients with unfavorable outcome (%)**
All TBI (n= 205)		
APOE ε4 allele	26 (17.4)	30 (53.6)
No *APOE* ε4 allele	123 (82.6)	26 (46.4)
Total	**149**	**56**
Mild (n= 41)		
APOE ε4 allele	12 (32.4)	2 (50.0)
No *APOE* ε4 allele	25 (67.6)	2 (50.0)
Total	**37**	**4**
Moderate (n= 92)		
APOE ε4 allele	12 (15.6)	11 (73.3)
No *APOE* ε4 allele	65 (84.4)	4 (26.7)
Total	**77**	**15**
Severe (n=72)		
APOE ε4 allele	4 (11.4)	17 (45.9)
No *APOE* ε4 allele	31 (88.6)	20 (54.1)
Total	**35**	**37**

Stratified by severity (Table 3.4), in the 72 patients with severe TBI (GCS < 9), 37 had unfavorable outcomes, 17 had at least one allele of *APOE* ε4, whereas, in the 35 patients with favorable outcomes, only 4 had at least one *APOE* ε4 allele. Thus, in severe TBI, there was a significant association between unfavorable outcome and possession of *APOE* ε4 allele (p= 0.01). In the 92 patients who had moderate TBI (GCS: 9-13) (Table 3.4), 15 had unfavorable outcomes, 11 of whom had at least one copy of the *APOE* ε4 allele, whereas, in the 77 patients with favorable outcomes, only 12 had at least one *APOE* ε4 allele. Thus, similar to severe TBI, there appear to be a significant association between unfavorable outcome and possession of *APOE* ε4 allele in moderate TBI (p= 0.01). All 6 patients who are homozygous for the ε4 allele had moderate TBI and unfavorable outcomes.

In the 41 patients with mild TBI (GCS: 14-15) (Table 3.4), 4 patients had unfavorable outcomes, with 2 having at least one copy of the *APOE* ε4 allele, whereas, in the 37 patients with favorable outcome, 12 patients had at least one *APOE* ε4 allele. In contrast to severe and moderate TBI, there seem to be no significant association between unfavorable outcome and *APOE* ε4 allele in mild TBI (p= 0.34).

We further explored the possibility that the *APOE* ε2 allele combined with *APOE* ε4 allele may mask the deleterious effects of *APOE* ε4 in TBI as previously described (S. D. Han et al., 2007; Nathoo et al., 2003; G. M. Teasdale et al., 1997). In 6 TBI patients in our cohort who had the ε2ε4 genotype, 5 (2 patients with mild TBI and 3 patients with moderate TBI) had favorable outcomes. Although the number of patients involved is small, if they were excluded from the statistical analysis, a stronger association between of *APOE* ε4 allele and unfavorable outcome (OR: 6.5, 95% CI: 3.2-13.2, p= 0.01) became apparent.

Analysis by the logistic model suggests that the GCS alone significantly predicts an unfavorable outcome in TBI (R^2 = 30.8, p=0.01). However, GCS paired with possession of an *APOE* ε4 allele is a better predictor for unfavorable outcome compared with GCS alone (R^2 = 60.9; *APOE* ε4 allele- OR: 8.0, 95% CI: 3.6-18.0, p= 0.01; GCS-OR: 5.0, 95% CI: 2.7-9.1, p= 0.01).

As for the -491 A/T, -427 C/T and -219 G/T polymorphisms in the *APOE* gene promoter, we did not find any association between unfavorable outcome and the presence of the -491 A allele, -427 C allele and -219 T alleles, or any other combinations (p > 0.01). We also did not find any association with unfavorable outcome when these polymorphisms were combined with possession of the *APOE* ε4 allele (p > 0.01).

3.5 Discussion

In this prospective cross sectional study, we found that *APOE* ε4 allele in patients with moderate and severe TBI were more likely to have an unfavorable global functional outcome at 6 months as measured with the GOS compared to patients without the *APOE* ε4 allele.

Evidence from several other studies support our finding that possession of the *APOE* ε4 allele is a genetic determinant for unfavorable outcome after TBI (Chiang et al., 2003; Friedman et al., 1999; Liaquat et al., 2002; Teasdale et al., 1997). Teasdale et al. (1997) was the first one to report this association in a prospective evaluation of 89 patients with TBI. The study reported that 17 (57%) of 30 patients with the *APOE* ε4 allele had a 2-fold increase in unfavorable outcome at 6 months compared with 16 (27%) of 59 patients without the allele. This first study provided the impetus to better understand the role of APOE gene in the setting of TBI as it may be essential in the assessment of prognosis. Moreover, patients with TBI could be counseled about the imminent risks from

injury and of cognitive decline in later life (G. M. Teasdale et al., 1997). Meanwhile, Chiang et al. (2003) studied 100 subjects with TBI and documented that 10 of 19 patients (52.6%) with the *APOE* ε4 allele had an unfavorable outcome compared with 20 of the 81 (24.1%) patients without the allele (Chiang et al., 2003). A study of 69 subjects by Friedman et al. (1999) also showed a similar trend in which 26 of 27 subjects (96.3%) with the *APOE* ε4 allele had an unfavorable outcome compared with 29 of 42 of those (69.0%) without the allele (Friedman et al., 1999). In addition, a recent meta-analysis (Zhou et al., 2008) indicated that although the presence of the *APOE* ε4 allele was not associated with the initial severity of brain injury after TBI, it was associated with increased risk of poor outcome at 6 months after injury. Most of these reports documented the effect of *APOE* ε4 on TBI outcome almost exclusively in white populations or those derived from Caucasians. The current study is the third from Asia to report an association between possession of *APOE* ε4 allele and unfavorable outcome after TBI. The other two studies mentioned above were from Israel (Friedman et al., 1999) and from Taiwan (Chiang et al., 2003).

There are a few studies which have focused on the outcome in patient subgroups with *APOE* ε4 (Han et al., 2007; Nathoo et al., 2003; Teasdale et al., 1997). Of the ten patients with ε2ε4 genotype in the study by Nathoo et al. (2003) six had a good outcome (Nathoo et al., 2003). In the present study, we found that out of 6 patients with ε2ε4 genotype 5 had good outcome. Although this appears to support the theory that *APOE* ε2 allele is neuroprotective, we were unable to show statistical significance based on the small number of patients.

The mechanisms underlying these associations are unclear at present but neuropathological studies have highlighted the importance of ApoE in the acute response to brain injury. Transgenic mice that express the human ApoE E4 isoform were associated with decreased synaptic plasticity (White, Nicoll, Roses, & Horsburgh, 2001), increased

mortality, increased size of brain injury and increased physical deficit compared to mice expressing the human ApoE E3 isoform (Sabo et al., 2000). In particular, neuronal plasticity was impaired over a 90-day period after entorhinal cortex lesion (White et al., 2001). Laskowitz et al. (2001) found that ApoE E4 isoform was less effective than either ApoE E2 or ApoE E3 at suppressing inflammatory cytokines in mouse and human microglial cultures (Laskowitz et al., 2001). ApoE isoform-specific brain inflammatory response was also recorded in transgenic mice expressing human ApoE E3 while ApoE E4 transgenic mice had a greater inflammatory response compared to ApoE E3 transgenic mice (Lynch et al., 2003). Isoform-specific effects of ApoE have also been demonstrated in neurite extension culture systems (Bellosta et al., 1995; DeMattos et al., 1998; Holtzman et al., 1995; Nathan et al., 1994; Sun et al., 1998). The observed inhibition of neurite extension by ApoE E4 appears to be related to alterations in the cytoskeleton, especially microtubule stability (Nathan et al., 1995). These effects may be mediated through tau (a microtubule stabilizing protein). ApoE E3, but not ApoE E4, binds to tau in vitro, and may protect tau from hyperphosphorylation, a process which inhibits tau's ability to stabilize microtubules (Lovestone et al., 1996; Strittmatter et al., 1994; Tesseur et al., 2000).

Nonetheless, there are a few studies that suggest that is no association between *APOE* ε4 allele and unfavorable outcomes at 6 months post-trauma (Alexander et al., 2007; Chamelian et al., 2004a; Diaz-Arrastia et al., 2003; Millar et al., 2003; Nathoo et al., 2003; Pruthi et al., 2010; Teasdale et al., 2005; Willemse-van Son et al., 2008). Teasdale et al, (2005) conducted one of the largest studies to date (U.K. based patient population) consisting of 1000 TBI patients. Their overall results revealed that 36% of *APOE* ε4 carriers had an unfavorable outcome (GOS 1, 2, 3) compared to 33% of non-carriers of *APOE* ε4 ($p=0.23$) at six-month follow-up. However, they found that

possession of the *APOE* ε4 allele in children and young adults was associated with unfavorable outcomes (Teasdale et al., 2005).

A study in an African population also failed to find a positive correlation between *APOE* ε4 allele and unfavorable outcome (Nathoo et al., 2003). Pruthi et al. (2010) also failed to find a positive correlation between presence of *APOE* ε4 allele and unfavorable outcome after TBI in 73 subjects from India, in which none of the 12 patients who had at least one *APOE* e4 allele had a poor outcome at six-month follow-up whereas 11(18%) patients without an *APOE* e4 allele had a poor outcome (Pruthi et al., 2010). Apparently, most of the studies which did not show association between unfavorable outcome and *APOE* ε4 allele either had only included mild and moderate TBI cases (excluding severe TBI) (Chamelian, Reis, & Feinstein, 2004b; Pruthi et al., 2010) or only included patients with contusion (Nathoo et al., 2003) or did not include death as an unfavorable outcome. In the current study we had included mild (GCS 15–14), moderate (GCS 13–9), and severe (GCS 8–3) cases (Teasdale & Jennett, 1974, 1976). Not surprisingly, mild TBI cases generally tend to have favorable outcomes whereas the severe TBI tend to have unfavorable outcomes. Thus, selectively excluding mild or severe TBI cases will bias the results toward unfavorable or favorable outcomes, respectively. We believe our study cohort which comprises all severity grades of TBI has less selection bias. We have also included death as an unfavorable outcome since excluding this cases could likewise selectively bias the results.

We did not find any association between polymorphisms of *APOE* promoter with unfavorable outcomes. In contrast, Lendon et al. (2003) in their study of 92 patients noted that poor recovery from TBI was more frequent among subjects with TT genotype of -219 G/T compared to the GG and GT genotypes (Lendon et al., 2003). Nevertheless, this study also reported absence of association between -491 A/T promoter polymorphism and unfavorable outcome (Lendon et al., 2003). Apart from our study, to date we are not aware

of any other study on the impact of -427 C/T promoter polymorphisms on outcome. The role of polymorphisms in the *APOE* promoter are still unclear and further research is needed to understand underlying mechanisms especially on how these polymorphisms alter the transcription and eventually affect the *APOE* gene itself in TBI patients.

Our findings and supportive evidence from other studies implies that in all TBI patients *APOE* genotype should be determined at admission especially in moderate/severe TBI. It may be a useful tool to alert clinicians to the possibility of a poorer outcome and therefore greater attention to these patients may be warranted (Ost et al., 2008; Teasdale et al., 1997). With further research into the mechanisms by which ApoE impacts the CNS's response to injury, ApoE may be a potential target for the development of future therapeutic intervention. Research findings supporting the use of ApoE and ApoE-derived peptide administration in the recovery process following TBI support this idea. ApoE seems to have a direct neuroprotective role, as neuronal damage in the entorhinal cortex and global ischaemia in *APOE* deficient mice is ameliorated by intraventricular infusion of ApoE (Graham, Horsburgh, Nicoll, & Teasdale, 1999; Horsburgh, McCarron, White, & Nicoll, 2000). Horsburgh et al. (2000) found that ApoE-deficient and ApoE wild-type mice with global ischemia has improved outcome when intrathecal administration of ApoE protein was given (Horsburgh, McCulloch, et al., 2000). Intraventricularly administered ApoE mimetic peptide derived from the receptor-binding region in postnatal day 7 rats immediately before hypoxic-ischemic injury showed less brain injury than control animals (McAdoo et al., 2005). Lynch et al. (2003) found similar results with intravenously administered ApoE mimetic peptide (residues 133–149) given 30 min after inducing TBI in mice. Animals receiving the peptide had improved functioning and reduction in the number of injured hippocampal neurons (Lynch et al., 2003).

One of the limitations of this study is the small number of females so we were unable to analyze if gender differences could affect the outcome after TBI, especially in those who had at least one *APOE* ε4 allele. Although wide inter-ethnic variations in the *APOE* gene and its promoter are thought to be responsible for inconsistent findings in the literature, our sample size does not have enough power to confidently confirm how ethnic differences in Malaysia could affect the outcome after TBI. Since this is the first study in a Malaysian population and the impact of *APOE* ε4 over extended periods are less clear, larger patient cohorts and experimental studies are needed to fully explain how *APOE* ε4 can unfavorably affect outcome after TBI.

3.6 Conclusion

Our findings show that presence of *APOE* ε4 allele is associated with poor outcome at 6 months after TBI, independent of polymorphisms in the promoter of the *APOE* gene. In our patients, GCS combined with *APOE* ε4 allele status were better predictors for unfavorable outcome at 6 months after TBI compared with GCS alone. Although, the prevalence of *APOE* ε4 allele among Asian is marginally less compared to the Caucasian and African population, we believe its deleterious role in TBI is supported by our findings. Thus, we propose that *APOE* genotyping, in addition to routine clinical management may aid clinician in targeting TBI patients that may need much more intensive care by:

- Providing adequate sedation that potentiates analgesics; provides anxiolysis; limits elevations of ICP related to agitation, discomfort, cough or pain; facilitates nursing care and mechanical ventilation; decrease O_2 consumption, $CMRO_2$, and CO_2 production; improves patient comfort; and prevents harmful movements.

- Regular monitoring that include electrocardiography (ECG monitoring), arterial oxygen saturation (pulse oxymetry, SpO_2), capnography (end-tidal CO_2, $PetCO_2$), arterial blood pressure (arterial catheter), central venous pressure (CVP), systemic temperature, urine output, arterial blood gases, and serum electrolytes and osmolality.
- ICP monitoring for earlier detection of intracranial mass lesion, guidance of therapy and avoidance of indiscriminate use of therapies to control ICP, drainage of CSF with reduction of ICP and improvement of CPP, and determination of prognosis.
- Controlling of systemic temperature as moderate systemic hypothermia at 32°C to 34°C, reduces cerebral metabolism and CBV, decreases ICP, and increases CPP.

CHAPTER 4

SINGLE NUCLEOTIDE POLYMORPHISMS IN GENES THAT MODULATE THE DOPAMINE SYSTEM: INFLUENCES ON 6 MONTH OUTCOME AFTER TRAUMATIC BRAIN INJURY

4.1 Introduction

Dopamine is an endogenous chemical that transmits signals from one neuron to another. It is the major neurotransmitter in the central dopaminergic pathways that plays a critical role in motor function (the nigro-striatal pathway), motivated behavior, hormonal stasis (the tubero-infundibular pathway), cognition (meso-cortical pathway), mood homeostasis and reward circuitry (the meso-limbic pathway). Dopamine may also be involved in neural plasticity and repair through effects on brain derived neurotrophic factor (Guillin et al., 2004), and in motor function recovery after TBI as seen in animal and human studies (Martinsson & Eksborg, 2004). Dopaminergic systems are carefully regulated in the CNS and are vulnerable to injury and dysregulation in TBI (Kobori et al., 2006; McAllister et al., 2004). Dysregulation could lead to increased levels of dopamine, which is a potently excitotoxic to the neurons. Therefore, genes that influence central dopaminergic function may play important roles in modulating the outcome of TBI (McAllister et al., 2004). Relatively little work has been done to study the association of SNPs in genes that modulate the dopamine system with outcome after TBI, particularly in Asian populations (Lipsky et al., 2005; McAllister et al., 2005). In this current study, we investigated if rs4680 (G/A) polymorphisms in the Catechol-O-Methyl Transferase gene, rs1800497 (C/T) polymorphisms of the Dopamine D2 receptor gene and rs6280 (C/T) polymorphisms in the dopamine D3 receptor gene could influence outcome in

patients with TBI. We hypothesize that there may be some SNPs that could impact on post-trauma recovery.

4.2 Literature Review

SNPs in genes that modulate the dopaminergic system have the potential to influence coding for dopamine receptor subtypes (DRD1- DRD5), dopamine re-uptake (the dopamine transporter, DAT) and dopamine metabolism (COMT). These SNPs are able to change a wide array of critical human functions. Many studies focused on finding SNPs in genes that modulate the dopamine system have been done. These include studies in neuropsychiatric disorders such as Parkinson's disease, and schizophrenia and its treatment with neuroleptics (M. F. Egan et al., 2001; Wong et al., 2000). The SNPs studied were rs4680 (G/A) polymorphisms in the Catechol-O-Methyl Transferase (*COMT*) gene, rs1800497 (C/T) polymorphisms of the Dopamine D2 receptor (*DRD2*) gene and rs6280 (C/T) polymorphisms in the dopamine D3 receptor (*DRD3*) gene as summarized in Table 4.1.

Table 4.1: SNPs in genes that modulate the dopamine system

GENE	CHROMOSOME LOCATION	ROLE	SNP	REFERENCES
COMT	22q11.21–23	Enzyme involved in breakdown of dopamine through the methylation of dopamine and norepinephrine	1. rs4680 (G/A) 2. The G allele codes for valine and A allele codes for methionine. 3. The enzyme with valine is almost four times as active as the enzyme with methionine at normal body temperature. 4. Under conditions of increased dopamine release, individuals with COMT A alleles may not able to breakdown dopamine efficiently. Increased level of dopamine, which is a potent excitotoxin to neurons may lead to less efficient neurotransmission and a worse performance.	(Egan et al., 2001; Lachman et al., 1996; Syvanen et al., 1997)
DRD2	11q22–23	Postsynaptic receptor for dopamine	1. rs1800497 (C/T) 2. Dopamine signaling at the dopamine D2 receptor can induce increases in intracellular Ca2+ release and activation of calcium dependent kinases and phosphatases important for cell death signaling 3. T allele has been associated with a 40% reduction in the expression of DRD2 receptors therefore individuals with T allele have better protection against cell death signaling.	(Azdad et al., 2009; Hernandez-Lopez et al., 2000; Pohjalainen et al., 1998)
DRD3	3q13.3	Postsynaptic receptor for dopamine	1. rs6280 (C/T). 2. The C allele code Glycine and T allele code for serine 3. DRD3 from the C allele confers an increased affinity for dopamine compared to the T allele 4. It is not known whether it affects outcome after TBI.	(Wong et al., 2000)

4.3 Method

This is a prospective cross sectional study of 205 hospitalized TBI patients from the same cohort described in Chapter 3 (3.3.1 Patient cohort, Page 37). The Glasgow Coma Score (GCS) on admission were determined as described (3.3.1 Patient cohort, Page 37) and Glasgow Outcome Scale (GOS) at six months was assessed as described (3.3.4 Outcome evaluation, Page 39). DNA was extracted from the patients' blood as previously described (3.3.2 DNA extraction and purification, Page 38). The polymorphisms in the *COMT* gene, the *DRD2* gene and the *DRD3* gene were genotyped with the TaqMan genotyping assay (Applied Biosystems, Foster City, CA). Statistical analysis is detailed in Chapter 3 (3.3.5 Statistical analysis, Page 39).

4.3.1 TaqMan SNP Genotyping Assays

The rs4680 (G/A) polymorphisms in the *COMT* gene, rs1800497 (C/T) polymorphisms of the *DRD2* gene and rs6280 (C/T) polymorphisms in the *DRD3* gene were genotyped with the TaqMan genotyping assay (Applied Biosystems, Foster City, CA) using the Real-time PCR 7500 Fast real-time polymerase chain reaction system (ABI7500 Fast; Applied Biosystems, Foster City, CA, USA). The assay-on-demand probes and primers were: C__25746809_50, C__7486676_10 and C__949770_10, for SNPs rs4680, rs1800497 and rs6280, respectively. The reaction volume was 10 μL, comprising 6 μL genotyping mix and 4 μL genomic DNA. The reaction conditions consisted of a pre-run at 95 °C for 10 min, following by 45 cycles of denaturation at 95 °C for 15 s and annealing step at 60 °C for 1 min.

4.4 Results

4.4.1 Genotype frequencies

Figure 4.1 shows the SNP genotyping scatter plots for the polymorphisms studied. In TaqMan SNP Genotyping Assays, the polymorphisms was determined by VIC/FAM fluorescence signal emitted by probes when it annealed and extended in the region of the interest in the DNA. If both alleles are homozygous either VIC or FAM fluorescence signal will be emitted by the probes. However if alleles are heterozygous both VIC and FAM fluorescence signal will be emitted together. The *COMT* homozygous G allele, *DRD2* homozygous C allele and *DRD3* homozygous C allele would emit FAM fluorescence signals only. The *COMT* homozygous A allele, *DRD2* homozygous T allele and *DRD3* homozygous T allele would emit VIC fluorescence signals only. Both VIC and FAM fluorescence signals would be emitted when *COMT*, *DRD2*, *DRD3* alleles are heterozygous.

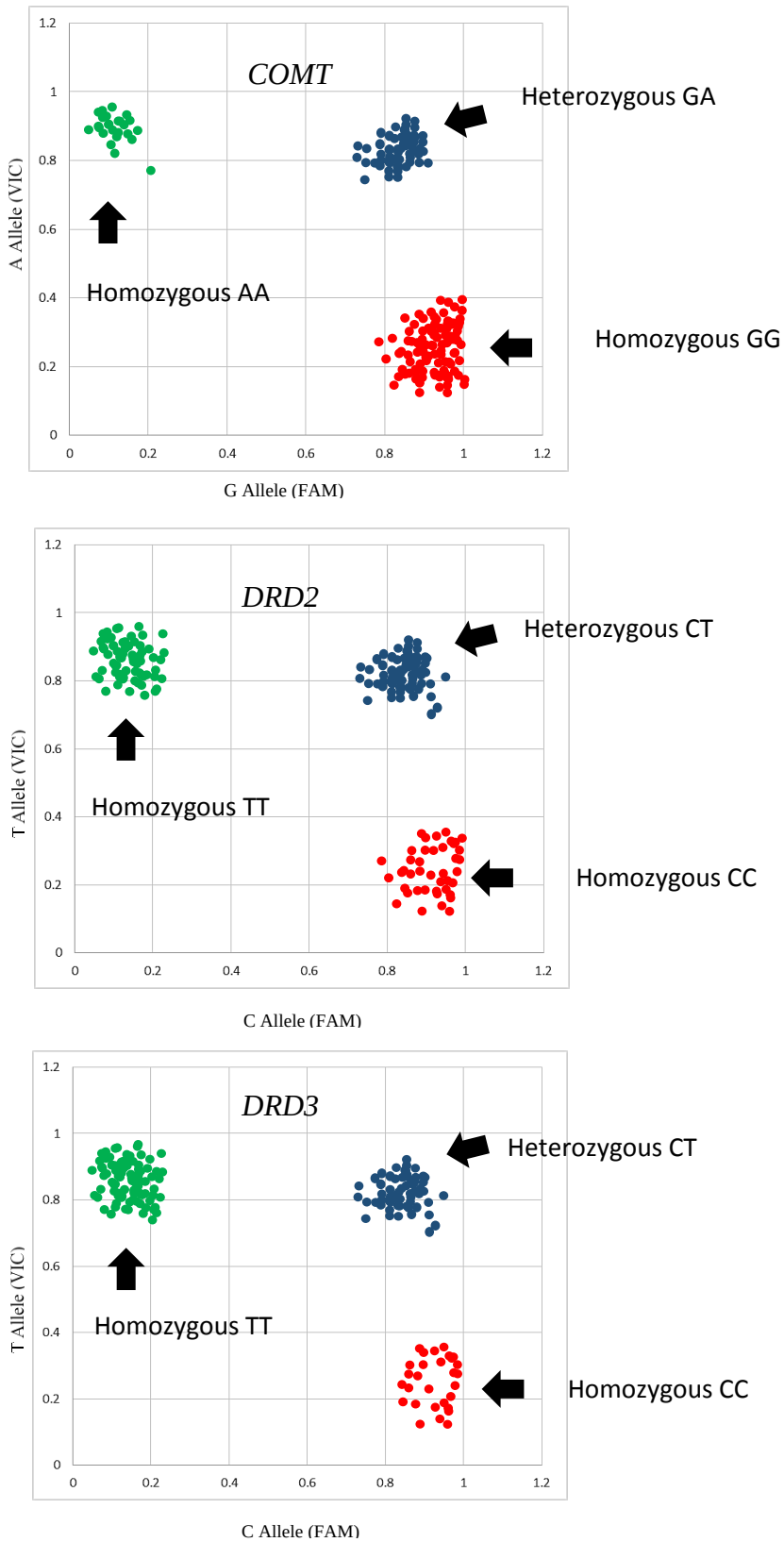

Figure 4.1: SNP genotyping scatter plot for rs4680 (G/A) polymorphism in the *COMT* gene, rs1800497 (C/T) polymorphism in the *DRD2* gene and rs6280 (C/T) polymorphism in the *DRD3* gene. Red dots show FAM signals indicating that both alleles are homozygous, green dots show VIC signals indicating that both alleles are homozygous and blue dots show VIC and FAM signals indicating that alleles are heterozygous.

The genotype frequencies for rs4680 (G/A) polymorphism in the *COMT* gene, rs1800497 (C/T) polymorphism in the *DRD2* gene and rs6280 (C/T) polymorphism in the *DRD3* gene in our study population are summarized in Table 4.2. For the rs4680 (G/A) polymorphism in the *COMT* gene, the most common and predominant genotype was homozygous GG found in 50.2% of subjects, followed by heterozygous GA genotype (38.6%) and homozygous AA genotype (11.2%). For the rs1800497 (C/T) polymorphism of the *DRD2* gene, 43.9% of the subjects were heterozygous CT. The rest were homozygous CC (20.5%) and homozygous TT (35.6%). For the rs6280 (C/T) polymorphism in the *DRD3* gene, the most common genotype was homozygous TT (46.3%), heterozygous CT (39.5%) and homozygous CC 14.2%.

Table 4.2: Genotype frequencies in 205 patients with TBI

	No. of patients	**%**
COMT		
GG	103	50.2
GA	79	38.6
AA	23	11.2
DRD2		
CC	42	20.5
CT	90	43.9
TT	73	35.6
DRD3		
CC	29	14.2
CT	81	39.5
TT	95	46.3

4.4.2 Outcome after TBI

Table 4.3 shows the frequencies of the COMT polymorphisms in patients further stratified by GCS. In general, the presence of *COMT* AA genotype was found to be significantly associated with an unfavorable outcome at 6 month after TBI (OR: 18.6, 95% CI: 5.97-58.03, p= 0.01). Out of the 56 patients who had unfavorable outcomes, 19 (33.9%) had the *COMT* AA genotype. In the 149 patients with favorable outcomes, only 4 (2.7%) had the *COMT* AA genotype. Nineteen (82.6%) out of 23 patients who had the *COMT* AA genotype had unfavorable outcomes. The various alleles of *DRD2* gene and *DRD3* gene were not associated with outcome after TBI (p > 0.01).

Table 4.3: *COMT* genotype and outcome in 205 patients with TBI

	No. of patients with favorable outcome (%)	No. of patients with unfavorable outcome (%)
All TBI (n= 205)		
COMT GG	85 (57.0)	18 (32.1)
COMT GA	60 (40.3)	19 (33.9)
COMT AA	4 (2.7)	19 (33.9)
Total	**149**	**56**
Mild (n= 41)		
COMT GG	23 (62.2)	1 (25.0)
COMT GA	14 (37.8)	1 (25.0)
COMT AA	0 (0)	2 (50.0)
Total	**37**	**4**
Moderate (n= 92)		
COMT GG	40 (51.9)	5 (33.3)
COMT GA	34 (44.2)	5 (33.3)
COMT AA	3 (3.9)	5 (33.3)
Total	**77**	**15**
Severe (n=72)		
COMT GG	22 (62.9)	12 (32.4)
COMT GA	12 (34.3)	13 (35.1)
COMT AA	1 (2.9)	12 (32.4)
Total	**35**	**37**

In the group of 72 patients with severe TBI (GCS <9), 37 had unfavorable outcomes, and 12 had the *COMT* AA genotype, whereas, in the 35 patients with favorable outcome, only 1 had the *COMT* AA genotype. Thus, there appear to be a significant association between unfavorable outcome and *COMT* AA genotype in severe TBI (χ^2 = 10.6, p= 0.01).

In the 92 patients who had moderate TBI (GCS: 9-13), 15 had unfavorable outcomes, 5 of whom had the *COMT* AA genotype, whereas, in the 77 patients with favorable outcomes, only 3 had the *COMT* AA genotype. Again, there appear to be a significant association between unfavorable outcome and *COMT* AA genotype in moderate TBI (χ^2 = 13.7, p= 0.01).

In the 41 patients with mild TBI (GCS: 14-15), 4 patients had unfavorable outcomes, with 2 having the *COMT* AA genotype, whereas, in the 37 patients with favorable outcome, none had the *COMT* AA genotype. Thus, there is also a significant association between unfavorable outcome and *COMT* AA genotype in mild TBI (χ^2 = 19.4, p= 0.01).

In the logistic model, GCS of the patients alone significantly predicts the unfavorable outcome in TBI (R^2 = 30.83, p=0.01). However, GCS paired with status of *COMT* A allele appears to be a better predictor for unfavorable outcome in TBI compared with GCS alone (R^2 = 60.61; *COMT* AA genotype- OR: 18.96, 95% CI: 5.48-65.56, p= 0.01; GCS- OR: 3.95, 95% CI: 2.17-7.17, p= 0.01).

4.5 Discussion

In this prospective cross sectional study of 205 patients with TBI, we have found that patients with the *COMT* AA genotype were associated with an unfavorable global functional outcome as measured by the GOS, for all grades of TBI. Unfavorable outcome after TBI was not associated with polymorphisms of *DRD2* and *DRD3* genes.

COMT A allele results in low activity of the COMT enzyme. Under conditions of increased dopamine release due to dysregulation of the dopaminergic system after TBI, individuals who have *COMT* AA genotype may not be able to breakdown high levels of dopamine which have accumulated in the synaptic clefts (Egan et al., 2001; Lubman et al., 2004; Syvanen et al., 1997). High levels of dopamine in the synaptic cleft are rapidly oxidized to form dopamine semiquinone/quinine. Oxidized dopamine *via* monoamine oxidase (MAO) activity or redox cycling can induce the generation of hydrogen peroxide and superoxide causing significant oxidative stress (Brunmark & Cadenas, 1988; Hastings, 1995; Olney et al., 1990; Sinet et al., 1980; Williams & Castner, 2006). Furthermore, increased dopamine signaling at the dopamine D2 receptor can increase intracellular Ca2+ release and activation of calcium dependent kinases phosphatases that are important for cell death signaling (Azdad et al., 2009; Hernandez-Lopez et al., 2000; So et al., 2009). This is supported by studies in animal models of TBI, which showed that following ischemia there is a 500 fold increase in dopamine concentration within the striatum (Globus et al., 1988). This increase precipitates excitotoxic disruption and oxidative damage to dopaminergic cellular function (Wagner et al., 2005). It is possible that individuals with *COMT* AA genotype are at a higher risk of excitotoxic and oxidative damage to their neurons after TBI. Significant neuronal damage may profoundly and unfavorably affect recovery after TBI.

To date, there is no other study which has associated specific SNPs of the *COMT* gene with functional outcome after TBI. However, a *COMT* gene study which evaluated cognitive performance or executive functioning of individuals with TBI and their response on the "Wisconsin Card Sorting Test" appeared to show results different from ours. Based on 113 individuals tested, individuals homozygous for A allele made fewer perseverative responses (good response) on the "Wisconsin Card Sorting Test" (Mean: 12.1 ± 5.1) than individuals homozygous for the G allele, who had the highest number of perseverative responses (mean: 20.0 ± 20.9). Heterozygotes made an intermediate number of perseverative responses (mean: 14.0 ± 10.9). Perseverative responding suggests problems with cognitive flexibility when the individual has difficulty shifting or changing mental set (Lipsky et al., 2005). However, one cannot determine from these data alone that TBI played a role in the observed association between *COMT* polymorphism and executive performance. The lack of a healthy comparison group in their study limits the ability to examine a causative relationship between *COMT* genotype and executive performance following TBI.

Failure to find an association between rs1800497 polymorphisms of *DRD2* gene and outcome after TBI is not entirely unexpected. Although studies have found that the T allele of rs1800497 was associated with poorer performance on measures of response latency ("Gordon Continuous Performance Test reaction times") and measures of episodic memory ("the California Verbal Learning Test - recognition trial") (McAllister et al., 2005), recent findings suggest that rs1800497 polymorphisms is strongly linked to another allele of an as yet undiscovered functional SNP located on the *DRD2* gene. In other words, every person with an rs1800497 T allele also has this undiscovered allele. Since we do not know of the presence of this allele, we end up mistakenly attributing causality to the rs1800497 T allele (McAllister, 2009). Further studies are needed to study

other SNP's of *DRD2* gene and its linkage with rs1800497 polymorphisms of *DRD2* gene and unfavorable outcome after TBI.

To date, there is no other study which associated specific SNP of *DRD3* gene with functional outcome after TBI. However, Bombin et al. (2008) evaluated 84 patients with first- episode psychosis with a comprehensive neuropsychological battery of test. The study assessed attention, working memory, memory and executive functions. Their findings suggest that subjects homozygous for *DRD3* C allele have poorer executive performance than subjects homozygous for T allele. This study did not find any significant differences in the other cognitive paradigms and postulated that rs6280 polymorphism of the *DRD3* gene could possibly to be involved solely with prefrontal cognition (Bombin et al., 2008). This might explain why no association was found between this polymorphism and executive functioning in our current study.

There are several limitations of this study that should be noted in interpreting the results. Outcome after TBI is a complex interaction of the profile and type of brain injury, effects of injury to other areas, post-injury treatment and various psychosocial factors. Thus the contribution of a single polymorphism to outcome could be quite modest. Another weakness in this study was the low number of females that does not allow us enough power to confirm that role of gender in outcome. Since this is the first study in a Malaysian population and the effects of the *COMT* AA genotype over extended longer period are less clear, further studies are needed to explain and confirm the association of *COMT* AA genotype with unfavorable outcome after TBI.

4.6 Conclusion

Our findings show that presence of *COMT* AA genotype is associated with poor outcome at 6 months after TBI. We also found that GCS paired with status of *COMT* AA genotype appears to be a better predictor for unfavorable outcome at 6 month after TBI compared with GCS alone. There is no association between alleles of *DRD2* gene and *DRD3* gene with outcome after TBI.

CHAPTER 5

SINGLE NUCLEOTIDE POLYMORPHISMS IN GENES THAT MODULATE THE NEUROTROPIC FACTORS: INFLUENCES ON 6 MONTH OUTCOME AFTER TRAUMATIC BRAIN INJURY

5.1 Introduction

Neurotrophic factors are small protein molecules that regulate growth and survival of neurons. They influence metabolic functions such as protein synthesis and the ability of the neuron to make the neurotransmitters for communication with other neurons or with other targets (e.g. muscles and glands). Hence, neurotrophic factors play a significant role in the maintenance of neuronal function throughout an individual's entire lifetime (Huang & Reichardt, 2001; Thoenen, 1995). Comparatively little work has been done to study the association between SNPs of neurotrophic factor genes with outcome after TBI, particularly in Asian populations. In the current study, we attempted to determine if rs1800169 (A/G) polymorphism of the ciliary neurotrophic factor gene (*CNTF*), rs6265 (A/G) polymorphism of the brain-derived neurotrophic factor gene (*BDNF*) and rs36119840 (A/G) polymorphism of the glial cell-derived neurotrophic factor gene (*GDNF*) could influence outcome in patients with TBI.

5.2 Literature Review

Neurotrophic factors can be grouped as Neurotrophins (Nerve Growth Factor, BDNF, neurotrophin-3, neurotrophin-4 and CNTF), Glial cell-line Derived Neurotrophic Factor family ligands (GDNF, neurturin, artemin, and persephin) and Neuropoietic Cytokines. Neurotrophic factors have been reported to contribute to the pathogenesis of some neurodegenerative disorders, and have been proposed as potential treatments for these diseases, based on evidence of in vitro experiments and animal model studies (Moris & Vega, 2003). Many genetic studies have focused on SNPs in neurotrophic factor genes in neuropsychiatric disorders such as Alzheimers's disease and schizophrenia (Huang et al., 2007; Lavedan et al., 2008; Voineskos et al., 2011; Xu et al., 2010) to identify SNPs that may be associated with increased risk for disease. These SNPs studies include rs1800169 (A/G) polymorphism of the *CNTF* gene, rs6265 (A/G) polymorphism of the *BDNF* gene and rs36119840 (A/G) polymorphism of the *GDNF* gene as summarized in Table 5.1.

Table 5.1: Neurotrophic factors: Characteristics, function and SNPs

GENE	CHARACTERISTICS AND FUNCTION	SNP	REFERENCES
CNTF	1. A member of the interleukin-6 family of cytokines 2. It can rescue various types of adult CNS neurons in disease models, including striatal, cholinergic forebrain, dopaminergic midbrain and motor neurons 3. CNTF stimulates gene expression, cell survival or differentiation in a variety of neuronal cell types such as sensory, sympathetic, ciliary and motor neurons 4. Nonneuronal cells, such as oligodendrocytes, microglial cells, liver cells, and skeletal muscle cells, respond to exogenously administered CNTF, both in vitro and in vivo	1. rs1800169 (G/A) 2. It is a null mutation of the *CNTF* gene. 3. The G to A transition introduces a new splice acceptor site and the resulting mRNA encodes a non- functional aberrant protein	(Anderson, Panayotatos, Corcoran, Lindsay, & Wiegand, 1996; Arakawa, Sendtner, & Thoenen, 1990; Barres et al., 1993; Dale, Kuang, Wei, & Varon, 1995; Emerich et al., 1997; Hagg, Quon, Higaki, & Varon, 1992; Hagg & Varon, 1993; Hagg, Varon, & Louis, 1993; Lo, Li, Oppenheim, Prevette, & Houenou, 1995; Louis et al., 1993; MacLennan et al., 1996; Mitsumoto et al., 1994; Pun, Santos, Saxena, Xu, & Caroni, 2006; Sagot et al., 1995; Sendtner et al., 1994; Sendtner, Kreutzberg, & Thoenen, 1990; Sendtner et al., 1992; Simon, Jablonka, Ruiz, Tabares, & Sendtner, 2010; R. Takahashi et al., 1994; Zala et al., 2004)
BDNF	1. BDNF is found abundantly in the hippocampus area 2. It plays an important role in memory, attention, and speed of information processing.	1. rs6265 (A/G) 2. The more common G allele encodes for valine, while the A allele encodes methionine. 3. The heterozygous form of this SNP produces a mature protein that has altered intrinsic biological activity.	(Egan et al., 2003; Farkas & Povlishock, 2007; Leibrock et al., 1989)

Table 5.1, Continued: Neurotrophic factors: Characteristics, function and SNPs

GENE	CHARACTERISTICS AND FUNCTION	SNP	REFERENCES
		4. This alteration affects intracellular processing and secretion of mature BDNF. 5. Therefore, compared to the homozygous BDNF form, neuroplastic effect of heterozygous BDNF may be compromised but the mechanism is unclear.	
GDNF	1. GDNF is a glycosylated, disulfide-bonded homodimer that is a distantly related member of the transforming growth factor-beta superfamily. 2. In embryonic midbrain cultures, recombinant human GDNF promoted the survival and morphological differentiation of dopaminergic neurons and increased their high-affinity dopamine uptake.	1. rs36119840 (A/G) 2. The role and function of this polymorphism and how it affects the protein is not clearly understood.	(Lin et al., 1993)

5.3 Method

This is a prospective cross sectional study of 205 hospitalized TBI patients from the same cohort described in Chapter 3 (3.3.1 Patient cohort, Page 37). The Glasgow Coma Score (GCS) on admission were determined as described (3.3.1 Patient cohort, Page 37) and Glasgow Outcome Scale (GOS) at six months was assessed as described (3.3.4 Outcome evaluation, Page 39). DNA was extracted from the patients' blood as previously described (3.3.2 DNA extraction and purification, Page 38). The polymorphisms in the *CNTF* gene, the *BDNF* gene and the *GDNF* gene were genotyped with the TaqMan genotyping assay (Applied Biosystems, Foster City, CA). Statistical analysis is detailed in Chapter 3 (Chapter 3; 3.3.5 Statistical analysis, Page 39).

5.3.1 TaqMan SNP Genotyping Assays

The rs1800169 (A/G) polymorphism of the *CNTF* gene, the rs6265 (A/G) polymorphism of the *BDNF* gene and the rs36119840 (A/G) polymorphism of the *GDNF* gene were genotyped with the TaqMan genotyping assay (Applied Biosystems, Foster City, CA) using the Real-time PCR 7500 Fast real-time polymerase chain reaction system (ABI7500 Fast; Applied Biosystems, Foster City, CA, USA), and assay-on-demand probes and primers: C__11592758_10, C__648651_20 and C__7511603_10, for SNPs rs6265, rs36119840 and rs1800169, respectively. The reaction volume was 10 μL, comprising 6 μL genotyping mix and 4 μL genomic DNA. The reaction conditions consisted of a pre-run at 95 °C for 10 min, following by 45 cycles of denaturation at 95 °C for 15 s and annealing step at 60 °C for 1 min.

5.4 Results

5.4.1 Genotype frequencies

Figure 5.1 shows the SNP genotyping scatter plots for the polymorphisms studied. The polymorphisms were determined by VIC/FAM fluorescence signals emitted by probes when they annealed and extended in the region of the interest in the DNA. If both alleles are homozygous, either VIC or FAM fluorescence signal will be emitted by the probes. However, if alleles are heterozygous, both VIC and FAM fluorescence signals will be emitted together. The *CNTF* homozygous A allele, *BDNF* homozygous A allele and *GDNF* homozygous A allele emit FAM fluorescence signals. The *CNTF* homozygous G allele, *BDNF* homozygous G allele and *GDNF* homozygous G allele emit VIC fluorescence signals. Both VIC and FAM fluorescence signals were emitted when *CNTF*, *BDNF*, *GDNF* alleles were heterozygous.

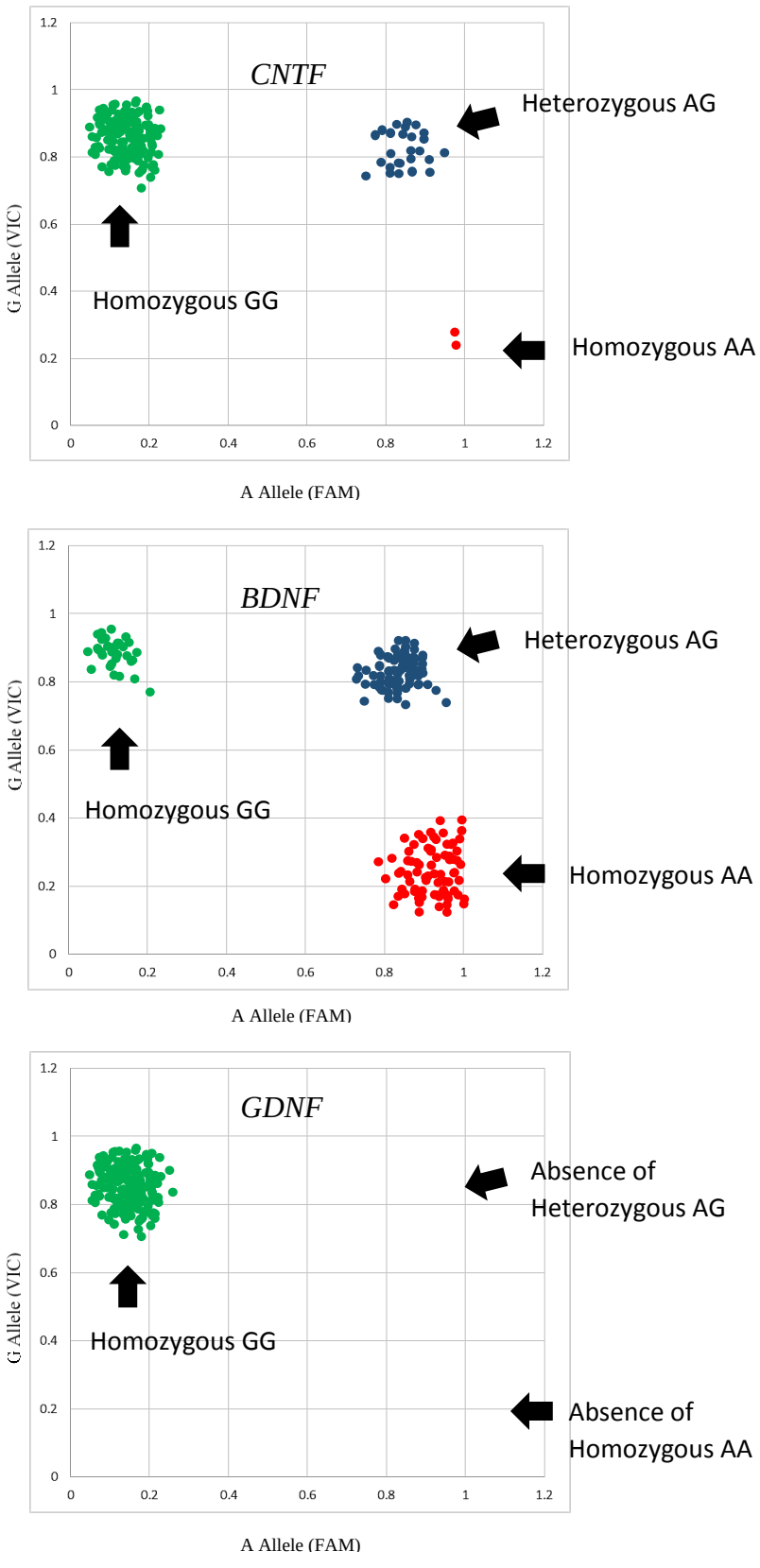

Figure 5.1: SNP genotyping scatter plot for rs1800169 (A/G) polymorphism of *CNTF* gene, rs6265 (A/G) polymorphism of *BDNF* gene and rs36119840 (A/G) polymorphism of *GDNF* gene.

Red dots show FAM signals indicating that both alleles are homozygous, green dots show VIC signals indicating that both alleles are homozygous and blue dots show VIC and FAM signals indicating that alleles are heterozygous.

The genotype frequencies for rs1800169 (A/G) polymorphism of *CNTF* gene, rs6265 (A/G) polymorphism of *BDNF* gene and rs36119840 (A/G) polymorphism of *GDNF* gene are summarized in Table 5.2. For the rs1800169 (A/G) polymorphism of the *CNTF* gene, the most common and predominant genotype was homozygous GG found in 76.1% of subjects, followed by heterozygous AG genotype (22.9%) and homozygous AA genotype (1.0%). For the rs6265 (A/G) polymorphism of the *BDNF* gene, 47.8% of the subjects were heterozygous AG, 37.6% homozygous AA and 14.6% homozygous GG. For the rs36119840 (A/G) polymorphism of the *GDNF* gene, the only genotype found was homozygous GG (100%); the *GDNF* homozygous AA and heterozygous GG genotypes were not found.

Table 5.2: Genotype frequencies in 205 patients with TBI

	No. of patients	**%**
CNTF		
AA	2	1.0
AG	47	22.9
GG	156	76.1
BDNF		
AA	77	37.6
AG	98	47.8
GG	30	14.6
GDNF		
AA	0	0
AG	0	0
GG	205	100

5.4.2 Outcome after TBI

The outcome after TBI was not associated with different alleles of *BDNF* gene and *GDNF* gene, respectively ($p > 0.01$). Table 5.3 shows the frequencies of the *CNTF* polymorphisms in patients further stratified by GCS. In general, for all the grades of TBI, the *CNTF* A allele is significantly associated with an unfavorable outcome at 6 months after TBI (OR: 3.7, 95% CI: 1.88-7.36, p= 0.01). Of the total of 205 patients, 56 had unfavorable outcomes, 24 (42.9%) of whom had at least one copy of the *CNTF* A allele, whereas, in the 149 patients with favorable outcomes, only 25 (16.8%) had at least one *CNTF* A allele (Table 5.3).

Table 5.3: *CNTF* genotype and outcome in 205 patients with TBI

	No. of patients with favorable outcome (%)	**No. of patients with unfavorable outcome (%)**
All TBI (n= 205)		
CNTF AA	2 (1.3)	0 (0)
CNTF AG	23 (15.4)	24 (42.9)
CNTF GG	124 (83.2)	32 (57.1)
Total	**149**	**56**
Mild (n= 41)		
CNTF AA	1 (2.7)	0 (0)
CNTF AG	6 (16.2)	3 (75.0)
CNTF GG	30 (81.1)	1 (25.0)
Total	**37**	**4**
Moderate (n= 92)		
CNTF AA	1 (1.3)	0 (0)
CNTF AG	12 (15.6)	7 (37.8)
CNTF GG	64 (83.1)	8 (62.2)
Total	**77**	**15**
Severe (n= 72)		
CNTF AA	- (0)	- (0)
CNTF AG	5 (14.3)	14 (37.8)
CNTF GG	30 (85.7)	23 (62.2)
Total	**35**	**37**

In the 72 patients with severe TBI (GCS <9), 37 had unfavorable outcomes, and 14 (37.8%) of these patients had at least one copy of the *CNTF* A allele, whereas, in the 35 patients with favorable outcome, only 5 (14.3%) had at least one *CNTF* A allele. There appear to be a significant association between unfavorable outcome in severe TBI and the *CNTF* A allele ($\chi^2 = 5.1$, p= 0.01).

In the 92 patients who had moderate TBI (GCS: 9-13), 15 had unfavorable outcomes, 7 (46.7%) of whom had at least one copy of the *CNTF* A allele, whereas, in the 77 patients with favorable outcomes, only 13 (16.9%) had at least one *CNTF* A allele. Again, there is a significant association between unfavorable outcome in moderate TBI and the *CNTF* A allele ($\chi^2 = 6.5$, p= 0.01).

In the 41 patients with mild TBI (GCS: 14-15), 4 patients had unfavorable outcomes, with 3 (75%) having at least one copy of the *CNTF* A allele, whereas, in the 37 patients with favorable outcome, only 7 (18.9%) patients had at least one *CNTF* A allele. Thus, there is a significant association between unfavorable outcome in mild TBI and *CNTF* A allele ($\chi^2 = 6.2$, p= 0.01).

In the logistic model, GCS of the patients alone significantly predicts unfavorable outcome in TBI ($R^2 = 30.83$, p=0.01). However, GCS paired with the presence of the *CNTF* A allele is a better predictor for unfavorable outcome in TBI compared with GCS alone ($R^2 = 46.15$; *CNTF* A allele- OR: 4.55, 95% CI: 2.10-9.86, p= 0.01; GCS- OR: 4.28, 95% CI: 2.42-7.58, p= 0.01).

5.5 Discussion

In this cross sectional study, we have found that patients with the *CNTF* A allele had an association with unfavorable global functional outcomes (as measured by the GOS), compared to patients without the *CNTF* A allele. Unfavorable outcomes after TBI were not associated with alleles of *BDNF* and *GDNF* genes.

In the setting of TBI, CNTF may be very vital for the regulation of excessive glutamate. Since TBI is associated with a massive release of glutamate (Bullock et al., 1998; Robertson et al., 2001), increased levels of extracellular glutamate could cause over-stimulation of glutamate receptors that may result in secondary events, leading to neuronal cell death (Floyd, Gorin, & Lyeth, 2005; Yi & Hazell, 2006). In fact, glutamate as the most abundant excitatory neurotransmitter in the brain could cause prolonged depolarization and subsequent ionic imbalance, ATP depletion and increases in intracellular free calcium levels that together culminate in cerebral edema, raised intracranial pressure (ICP), vascular compression and brain herniation (DeWitt & Prough, 2003; Obrenovitch & Urenjak, 1997). Following TBI, CNTF-activated astrocytes aid in buffering the effects of glutamate in the CNS by enhanced glutamate uptake (Beurrier et al., 2010). However, in individuals with the *CNTF* A allele, a non- functional aberrant CNTF protein is produced. Hence, it is likely that individuals with the *CNTF* A allele are at higher risk of excitotoxic damage to their neurons after TBI, contributing to unfavorable post-TBI recovery.

In contrast to other studies we did not find any association between SNPs of the *BDNF* gene and outcome after TBI. Krueger et al. (2011) evaluated the effect of SNPs in the *BDNF* gene on executive function following TBI, and showed that G allele (valine) carriers had impaired executive function compared to the A allele (methionine) carriers (Krueger et al., 2011). The authors speculated that the A allele may favorably protect

neurons from apoptosis after injury through reduction of proBDNF secretion. Although BDNF helps to promote cell survival, its premature form, proBDNF, (Chao, 2003) tends to trigger apoptosis (R. Lee, Kermani, Teng, & Hempstead, 2001; Teng et al., 2005). Since, the A allele is associated with reduced secretion of proBDNF (Chiaruttini et al., 2009), it may favorably protect neurons from apoptosis after injury and eventually prevent declines in executive function following TBI.

There are several limitations in this study that should be noted in interpreting the results. Outcome after TBI is a complex interaction of the profile and type of brain injury, effects of injury to other areas, post-injury treatment and various psychosocial factors. Thus the contribution of a single polymorphism to outcome is probably quite modest. Another weakness in this study was the small number of females so this study did not have enough power to confirm that role of gender in TBI outcome. Since this is the first study in Malaysian population and the effects of *CNTF* A allele over a longer period are less clear, further and larger cohort studies are needed to explain why the *CNTF* A allele is associated with unfavorable outcome after TBI.

5.6 Conclusion

Our findings showed that presence of *CNTF* A allele is associated with poor outcome at 6 month after TBI. We also found that GCS paired with status of *CNTF* A allele are a better predictor for unfavorable outcome at 6 months after TBI compared with GCS alone. There is no association between alleles of *BDNF* gene and *GDNF* gene, respectively, with outcome after TBI.

CHAPTER 6

SERUM BIOMARKERS ASSOCIATED WITH TRAUMATIC BRAIN INJURY

6.1 Introduction

Serum biomarkers have assisted physicians to identify and assess injuries to various organs e.g. “troponin and creatine phosphokinase isoenzyme MB for the heart, aspartate and alanine transaminases for the liver, lipases for pancreas, and blood urea nitrogen and creatinine for the kidney”. Similarly in TBI, several putative serum biomarkers have been used but they suffer from a lack of specificity in that they can be released into the serum by conditions not directly due to TBI. Moreover, serum biomarkers to determine the severity of TBI (mild, moderate and severe) have not been well studied.

Conventional methods to study biomarkers for diseases/conditions had used ELISA and 2D gel-based mass spectrometry. In the last decade, mass spectrometry-based proteomics using iTRAQ technology for quantification has emerged as a new method for large-scale analysis of proteins in many types of diseases (Schulze & Usadel, 2010). As far as we are aware, so far there is only one study that used iTRAQ-based mass spectrometry to identify serum biomarkers in TBI (Hergenroeder et al., 2008). In this study, pooled sera of 11 cases of TBI were compared with sex and age matched normal controls and 15 candidate biomarkers were found to be increased; an additional 16 biomarkers were found to be decreased in severe TBI. However, the association of these proteins with mild and moderate TBI were not investigated.

The objectives of this study were to identify serum proteins which may be increased or decreased after TBI alone by iTRAQ-based mass spectrometry, and to evaluate if these serum proteins were able to distinguish mild, moderate and severe TBI. We hypothesize that changes in multiple serum proteins may provide the specificity and sensitivity required to diagnose TBI in the setting of polytrauma, and may aid in the timely diagnosis of TBI in a minimally invasive manner.

6.2 Literature Review

The GCS provides a prognostic scale for TBI (Teasdale & Jennett, 1974, 1976). Due to its simplicity, reproducibility and prognostic ability (albeit limited) it has become a standard component of the initial examination of head injury patients. However, given the increased use of intubation, ventilation and sedation of patients with impaired consciousness (Marion & Carlier, 1994), the utility of GCS becomes limited since the patients are often under the influence of sedative medication by the time they arrive at the trauma unit or even before arrival at the hospital. In these cases, assessing GCS at this time will not be indicative of the actual conscious state. In a study of 1005 patients with TBI in European centers, assessment of each of the three components of the GCS (eye opening, motor and verbal response) was possible only in 61% of patients before hospital arrival, in 77% on arrival at the first hospital, in 56% on arrival in the neurosurgical unit, and in 49% of post-resuscitation patients (Murray et al., 1993). In addition, factors like drug use, alcohol intoxication, shock or low blood oxygen also can alter a patient's level of consciousness which could eventually lead to an inaccurate GCS score. Problems also arise when the periorbital region is swollen, either following periorbital edema, direct ocular trauma, facial injury, craniotomy, cranial nerve VII injury or neuromuscular blockade. Mild TBI may be present in patients who appear normal with full GCS scores,

and a neuroimaging investigations such as the CT scan or brain MRI may or may not show evidence of any damage.

Hence, a good serum biomarker for TBI could complement the clinical assessment of the conscious state of patients. It may perhaps be the sole measure of the severity of TBI in patients who have altered conscious states such as in patients with sedative drugs or hypotension or who are under the influence of alcohol. The application of serum protein markers could potentially lead to expeditious grading in the case of intubated, sedated and unconscious patients even before the application of neuroimaging techniques. To date, the most frequently studied TBI-related serum markers, and some of their characteristics are summarized in Table 6.1. (Full details are found in chapter 2, page 25 to page 34). As indicated, most of them suffer from relative disadvantages as a serum biomarker for TBI.

Table 6.1: Most frequently examined TBI related serum markers and their relative disadvantages

SERUM MARKER	DISADVANTAGES	REFERENCES
S100B	– Elevated after bone fractures, thoracic contusions without fractures, burns and even after minor bruises – Elevated in local ischemia and reperfusion of the liver, gut, and kidney	(Anderson et al., 2001; Pelinka, Harada, et al., 2004)
Glial Fibrillary Acidic Protein	Not sensitive enough for mild TBI	(Vos et al., 2004)
Neuron Specific Enolase	Elevated in neuroendocrine bladder tumors small cell lung cancer, neuroblastoma and stroke.	(Schoerkhuber et al., 1999)
Myelin Basic Protein	Released into the bloodstream under the circumstances of demyelinating disease	(Ingebrigtsen & Romner, 2002)
Creatine Kinase Brain Isoenzyme	– Has short half-life and rapidly eliminated from circulation – Blood brain barrier disruption is necessary for it to be present in serum. – Elevated during adenocarcinomas of the prostate, ovary, colon, breast, small cell anaplastic carcinoma of the lungs and other adenocarcinomas of the gastrointestinal tract making it not specific for TBI.	(Schwartz et al., 1989)
Cleaved tau	Poor diagnostic value for mild TBI	(Zemlan et al., 2002)
C- Reactive Protein & Serum Amyloid A	General injury markers	(Hergenroeder et al., 2008)
Interleukin 6	General marker for multi-organ failure	(Chiaretti et al., 2008; Winter, Pringle, Clough, & Church, 2004)
Interleukin 8	General marker of inflammation	(Buttram et al., 2007)
Interleukin 10	General anti-inflammatory marker	(Bell et al., 1997; Csuka et al., 1999)
Interleukin 12p70	General marker of inflammation	(Buttram et al., 2007)
Tumor Necrosis Factor α	General marker of inflammation	(Crespo et al., 2007)

6.3 Methods

6.3.1 Sample Collection

For this study we used another cohort of TBI patients which is separate from the original cohort of 205 patients. Blood samples from 10 patients per group were collected from mild, moderate and severe TBI patient groups, respectively after obtaining consent. From each patient 10 mls of blood were drawn from the patient within 24 hours of admission to the Neuro Intensive Care Unit, University of Malaya Medical Centre. In this way, Each TBI group comprised samples from 5 males and 5 females. Their ages and nature of accidents summarized in Table 6.2, were generally representative of the cases in our original cohort of 205 patients. Age and sex-matched control samples were also obtained from 10 healthy individuals.

Patients who had blood transfusions, and who had co-morbidities such as extra-CNS traumatic injuries, polytrauma (e.g. fractures, abdominal and chest injuries), and other major systemic diseases that might interfere with serum protein marker discovery, were excluded from the study. The bottles were allowed to stand upright at room temperature for 1 hour for the clot to form. Upon centrifuging (2500 RPM, 4˚C) the serum separated and stored at -80 ˚C for subsequent analysis. Sera from each TBI group were then pooled for analysis with mass spectrometry.

Table 6.2: Characteristics of TBI groups for mass spectrometry

	Severity of TBI		
	Mild (n=10)	Moderate (n=10)	Severe (n=10)
Nature of accident			
MVA			
Motorcycle	3	5	7
Car	2	1	-
Other Vehicles	1	1	-
Pedestrian	-	-	2
Fall	3	1	-
Fall from a height	1	-	1
Assault	-	2	-
Gender			
Male (n=15)	5	5	5
Female (n=15)	5	5	5
Age			
16-30 (n=12)	4	4	4
31-45 (n=10)	4	4	4
>45 (n=8)	2	2	2

Abbreviation: Motor Vehicle Accident (MVA); Footnote: For the subheading MVA, patient was using the vehicle when met with an accident.

6.3.2 iTRAQ Reagent- labeling

Four iTRAQ (Isobaric tag for relative and absolute quantitation; AB SCIEX, USA) reagents (114, 115, 116 and 117) were utilized to label the primary amines of peptides in each of the 4 groups (mild, moderate, severe TBI and normal control groups), respectively. Each iTRAQ molecule consists of a reporter group linked to a peptide reactive group (Figure 6.1). The peptide reactive group binds to the primary amines of the peptide of interest to facilitate its quantification via measurement of reporter signal intensities by the "5800 MALDI TOF/ TOF mass spectrometer" (Applied Biosystems, USA). Duplicate sets from each of the four serum pools (mild TBI, moderate TBI, severe TBI, and normal control groups) were immunodepleted of the top 14 high-abundance proteins using the "Agilent MARS14 column" (Agilent, USA). Immunodepleted sera were "reduced", "alkylated", "trypsin digested" and "labelled with iTRAQ® reagent" according to the manufacturer's protocol (AB SCIEX, USA).

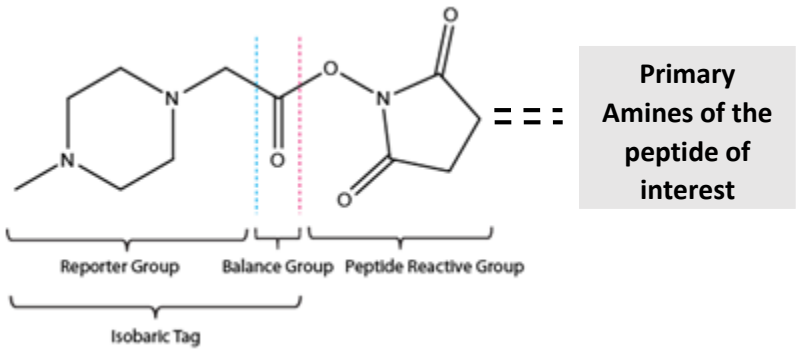

Figure 6.1: Structure of iTRAQ reagent- modified from Ross et al. (2004)

iTRAQ reagent consist a reporter group with mass=114, 115, 116, or 117 and a peptide reactive group that is reactive and linked to the primary amines of the peptide of interest.

6.3.3 First Dimension Ion Exchange Chromatography

The first dimension ion exchange chromatography was utilized to separate serum proteins according to their net charge. To achieve this, iTRAQ labeled peptides from the pooled sera were desalted on a "Strata-X 33 μm polymeric reversed phase column" (Phenomenex) and dissolved in a buffer containing 10 mM potassium hydrogen phosphate, pH 3 in 10% acetonitrile, before separation by "strong cation exchange liquid chromatography" (SCX) on an "Agilent 1100 HPLC system" (Agilent Technologies, USA) using a "PolySulfoethyl column" (4.6 x 100 mm, 5μm, 300 Å, Nest Group, USA). Peptides were eluted with a linear gradient of 0-400 mM KCl. A total of 8 fractions were collected, desalted on a "Strata-X 33 μM polymeric reversed phase column".

6.3.4 Second Dimension Reverse Phase nano- Liquid Chromatography

Eluted fractions were subjected to the second dimension reverse phase nano- liquid chromatography that uses a hydrophobic stationary phase to separate the proteins by size. The fractions were loaded onto a "C18 pre-column" and then separated on a "C18 PepMap100, 3 μm column" (Dionex, USA) using the "Ultimate 3000 nano HPLC system" (Dionex, USA). Peptides were resolved with a gradient of 10%- 40% acetonitrile (0.1% trifluoroacetic acid) and fractions were spotted every 30 seconds to "AnchorChip MALDI plates" using "ProBot robotic spotter" (LC Packings)

6.3.5 Matrix-Assisted Laser Desorption Ionization- Mass Spectrometry (MALDI-MS/MS)

The MALDI is a soft ionization technique in which a peptide sample is vaporized from a solid phase directly into a gas phase, ionized and accelerated to separate the ions according to their masses. Spotted chips containing peptide fractions were analyzed in a "5800 MALDI TOF/ TOF mass spectrometer" (Applied Biosystems, USA) operated in reflector positive mode. Mass spectrometry (MS) data were acquired over a mass range of 800−4000 m/z, and for each spectrum, a total of 400 shots were accumulated. A job-wide interpretation method selected the 20 most intense precursor ions above a signal/ noise ratio of 20 from each spectrum for MS/MS acquisition but only in the spot where their intensity was at its peak. MS/ MS spectra were acquired with 4000 laser shots per selected ion with a mass range of 60 to the precursor ion −20.

6.3.6 Data Analysis

Protein identification was performed using the "ProteinPilot 4.0 Software" (Applied Biosystems, USA). MS/MS spectra were searched against the "Swiss-Prot" human genomic database using search parameters as follows: "Cys alkylation, MMTS; Digestion, trypsin; Instrument, 5800; Special factors, none; Species, none; iTRAQ 4plex (peptide labeled) modification; Quantitate tab, checked; Detected protein threshold (unused ProtScore), 1.3, which corresponds to proteins identified with >95% confidence".

For quantitation analysis, the duplicates from each of the 4 pools serum pools were analyzed separately. Average protein ratios and p-values to indicate significant increase or decrease were calculated by the software. To be considered increased or decreased, proteins were required to have an unused protein score greater than 1.3,

corresponding to a confidence interval of 95%, and to have significantly different protein ratios in both duplicates (≥1.6 is increased, <0.625 is decreased), also at a confidence level of 95% ($p < 0.05$). The p values represented the variation in the reported iTRAQ ratios for all the peptides of the associated protein and do not relate to either biological variation or technical reproducibility. The false discovery rate was less than 1%, calculated using a database containing reversed sequences. In order to categorize the identified proteins, the results were analyzed using the software program IPA (Ingenuity Databases) and the UniProt Database release 2011_6 (http://www.uniprot.org/).

Figure 6.2 summarizes the workflow to prepare samples for mass spectrometry.

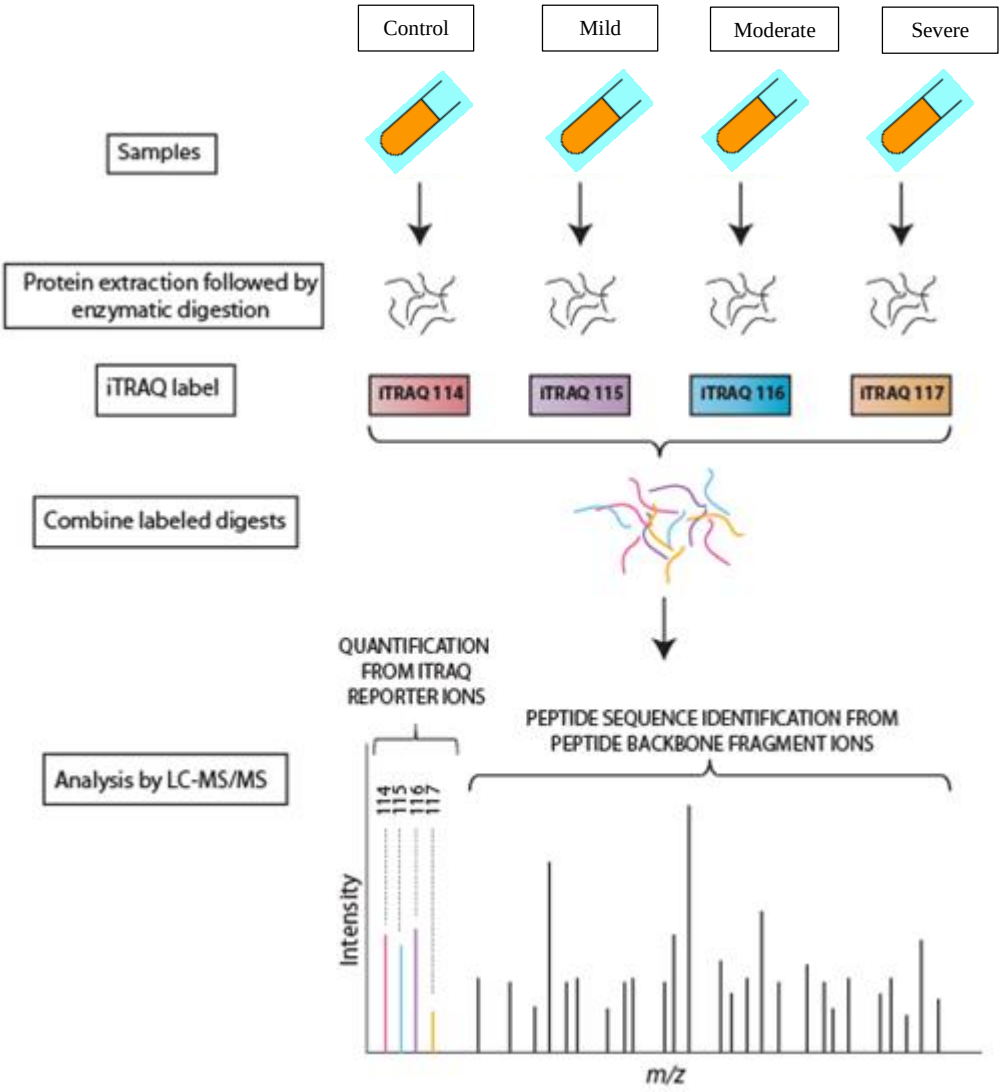

Figure 6.2: iTRAQ workflow- modified from (Ross et al., 2004)

Pooled samples from control, mild, moderate and severe TBI groups were digested and iTRAQ labelled for mass spectrometry. Combined labelled digests were chromatographically separated by charge and size, and analyzed by the mass spectrometer. Peptide sequence identification was derived from the spectra chart that showed various peptide sequences as vertical peaks. Quantification of the protein was obtained from the iTRAQ reporter ion signal intensities.

6.3.7 Validation with ELISA

Proteins which were identified to be increased or decreased by iTraq were confirmed and validated by commercially-available ELISA in accordance with the manufacturers' instruction (ABCAM, United Kingdom). ELISA was performed on entirely new groups of normal controls, mild, moderate and severe TBI patient samples (n=10 for each group), which were collected separately from those used for mass spectrometry. These samples were also age and sex matched to patients in the mass spectrometry study. In addition to normal controls (n=10), blood samples were also collected from another group of 10 patients with femoral fractures as additional controls. Data were expressed as mean ± standard deviation and statistical analysis was performed with SPSS 13.0 software (SPSS, Chicago, IL). $p < 0.05$ was considered statistically significant increase/decrease.

6.4 Results

The spectra from mass spectrometry analysis identified a total of 83 proteins (Appendix H) with >95% confidence in each of the 3 TBI group pooled sera samples. From this list, we identified 12 proteins that in general have a consistent and proportionally/gradually increasing or decreasing serum levels in the 3 TBI groups. Using these protein names as keywords, we extensively searched existing published literature available in Pubmed, EBSCO and Web of Science databases for links to neurological diseases/conditions. In this way, 11 proteins that have some relationship to brain pathology were shortlisted; 1 protein ("Ceruloplasmin") was excluded for lack of any such relationship. The spectra of these 11 proteins of particular interest are shown in panels A of Figure 6.3 to Figure 6.13. These proteins include:

1. "Serum Amyloid-A" (Figure 6.3)
2. "C- Reactive Protein" (Figure 6.4)
3. "Leucine- Rich alpha-2 Glycoprotein-1" (Figure 6.5)
4. "Lipopolysaccharide Binding Protein" (Figure 6.6)
5. "Fibronectin" (Figure 6.7)
6. "Vitronectin" (Figure 6.8)
7. "Alpha-1 antichymotrypsin" (Figure 6.9)
8. "Apolipoprotein E" (Figure 6.10)
9. "Zinc Alpha-2 glycoprotein" (Figure 6.11)
10. "Gelsolin" (Figure 6.12)
11. "Kininogen" (Figure 6.13)

In the spectra, various peptide sequences of a particular protein, shown as vertical peaks, enable the positive identification of the protein. For an example, panel A in Figure 6.3 shows the peptide sequence SFFSFLGEAFDGAR as denoted in the "Swiss-Prot" protein database, thus identifying the protein as "Serum Amyloid-A".

Panel B in Figure 6.3 to Figure 6.13 show the relative abundance of proteins in each of the normal control, mild, moderate and severe TBI groups. For an example, in panel B, Figure 6.3, the 4 peaks indicate the relative abundance of "Serum Amyloid-A" in all 4 groups, the highest being in the severe TBI group. Conversely, in panel B, Figure 6.12, the 4 peaks indicate that "Gelsolin" in the 4 groups, were almost similar in relative abundance.

Protein levels were only considered to be increased in sera from TBI subjects if the iTRAQ ratio of the relative abundance of the protein in TBI sera to normal control sera was ≥ 1.6. If the iTRAQ ratio of TBI/control was <0.625, then the protein was considered to be decreased. These cut-off ratios are equivalent to a confidence interval of 95% ($p < 0.05$).

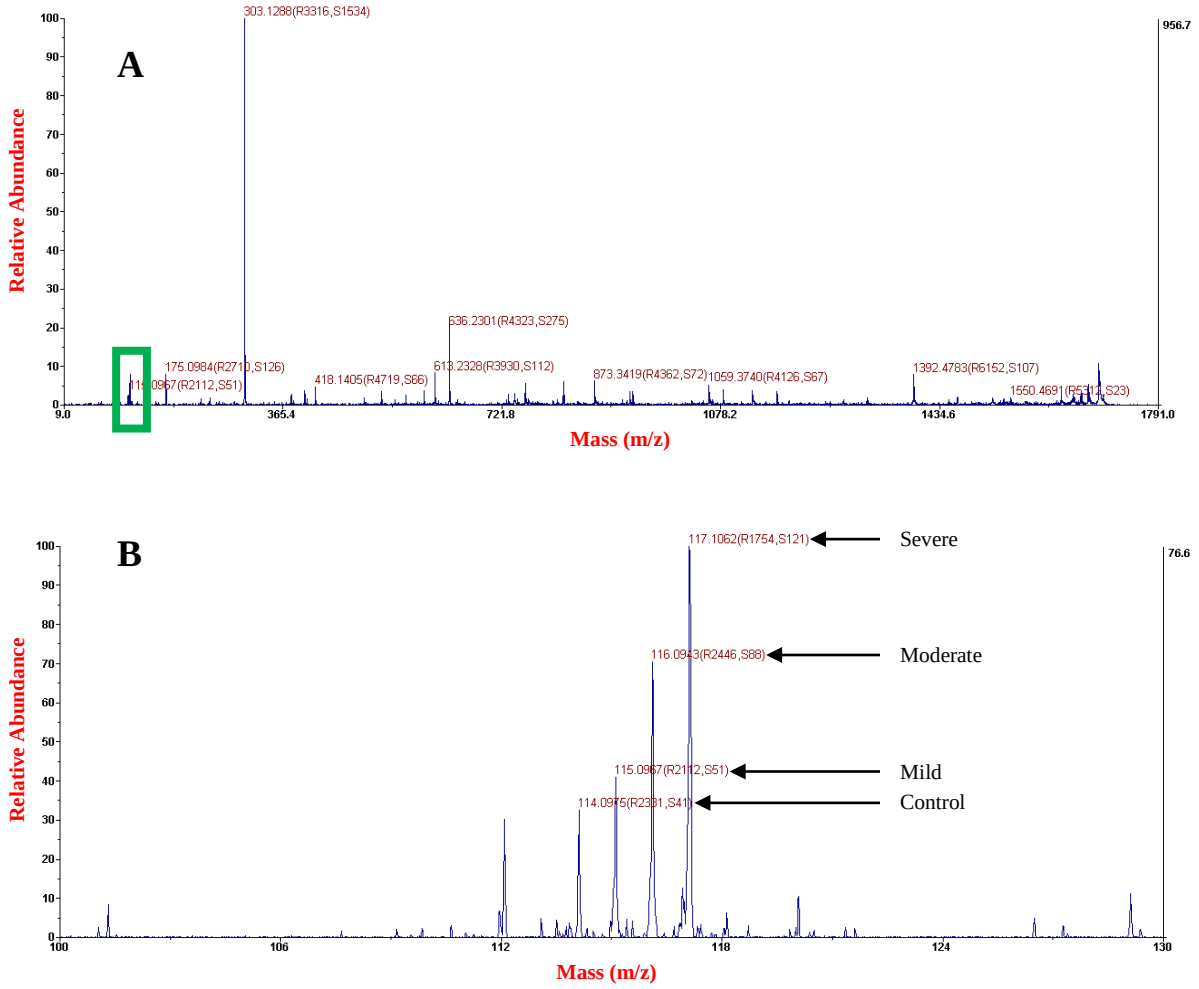

Figure 6.3: MS data acquired for "Serum Amyloid-A".

Panel A shows the multiple peaks of the protein spectrum of "Serum Amyloid-A" as identified by the "Protein Pilot 4.0 Software" using the peptide sequence SFFSFLGEAFDGAR in the "Swiss-Prot" protein database. These peaks were derived from the sera pools of normal control, mild, moderate and severe TBI groups. The green box (bottom left of panel A) shows iTRAQ signals from each of the 4 groups and are reproduced in Panel B as an enlarged image. The 4 peaks in Panel B indicate the levels of "Serum Amyloid-A" protein in these groups. Severe TBI (ratio over control= 47.9) had the highest level of the protein, followed by moderate (ratio over control= 35.3) and mild TBI (ratio over control= 29.4). A ratio of ≥1.6 is considered as a significant increase.

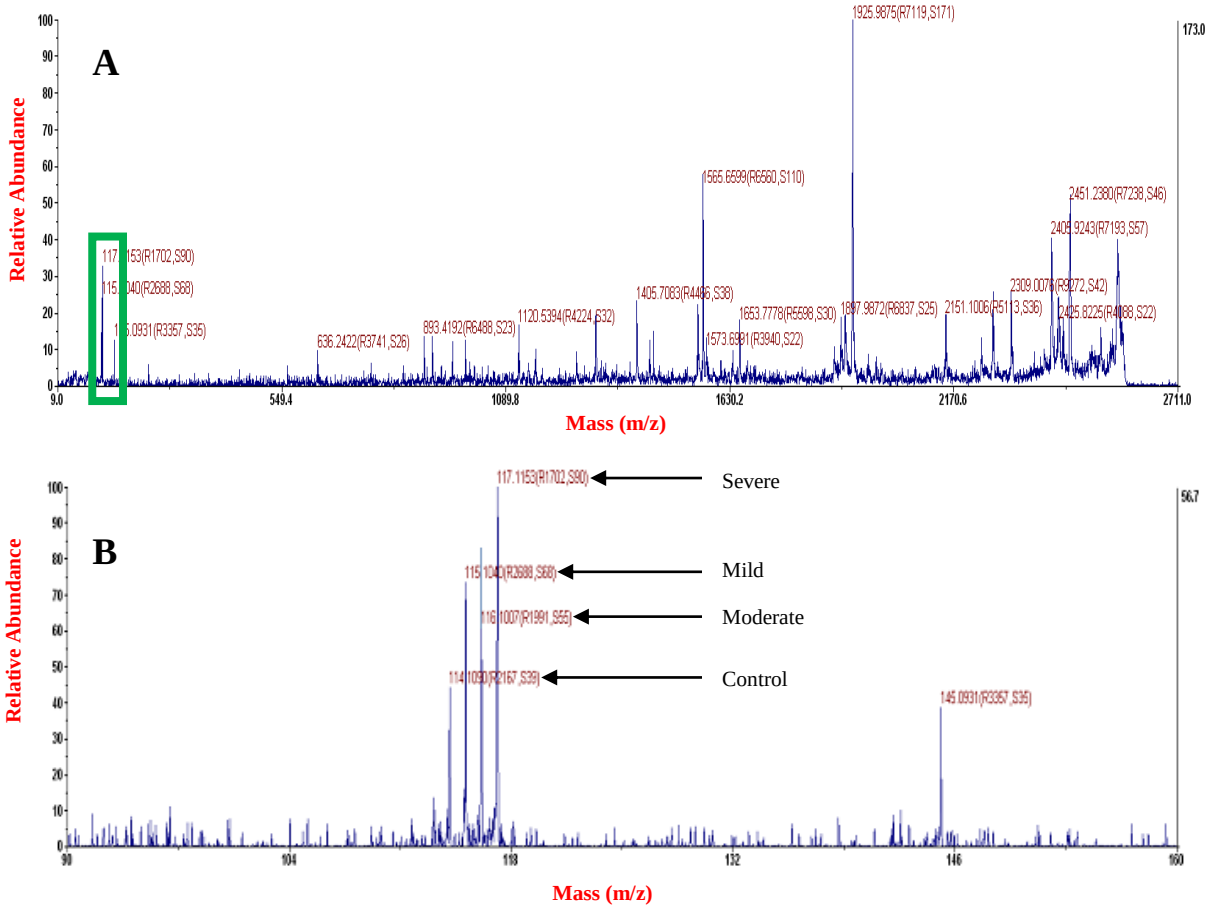

Figure 6.4: MS data acquired for "C-reactive protein".

Panel A shows the multiple peaks of the protein spectrum of "C-reactive protein" as identified by the "Protein Pilot 4.0 Software" using the peptide sequence ALKYEVQGEVFTKPQLWP in the "Swiss-Prot" protein database. These peaks were derived from the sera pools of normal control, mild, moderate and severe TBI groups. The green box (bottom left of panel A) shows iTRAQ signals from each of the 4 groups and are reproduced in Panel B as an enlarged image. The 4 peaks in Panel B indicate the levels of "C-reactive protein" protein in these groups. Severe TBI (ratio over control= 32.0) had the highest level of the protein, followed by moderate (ratio over control= 20.0) and mild TBI (ratio over control= 16.7). A ratio of ≥1.6 is considered as a significant increase.

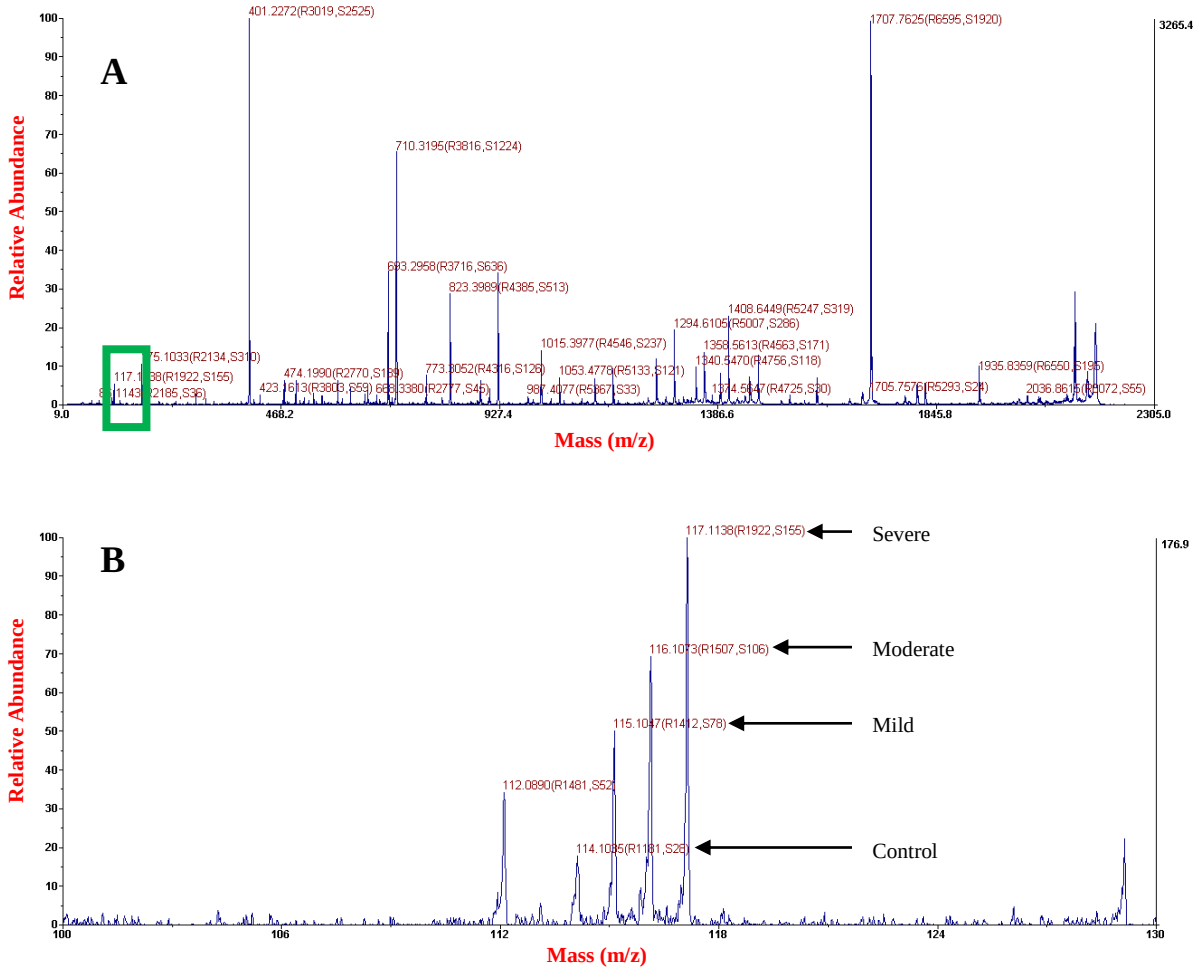

Figure 6.5: MS data acquired for "Leucine-rich alpha-2 glycoprotein-1".

Panel A shows the multiple peaks of the protein spectrum of "Leucine-rich alpha-2 glycoprotein-1" as identified by the "Protein Pilot 4.0 Software" using the peptide sequence TLDLGENQLETLPPDLLR in the "Swiss-Prot" protein database. These peaks were derived from the sera pools of normal control, mild, moderate and severe TBI groups. The green box (bottom left of panel A) shows iTRAQ signals from each of the 4 groups and are reproduced in Panel B as an enlarged image. The 4 peaks in Panel B indicate the levels of "Leucine-rich alpha-2 glycoprotein-1" protein in these groups. Severe TBI (ratio over control= 37.0) had the highest level of the protein, followed by moderate (ratio over control= 23.8) and mild TBI (ratio over control= 21.3). A ratio of ≥1.6 is considered as a significant increase.

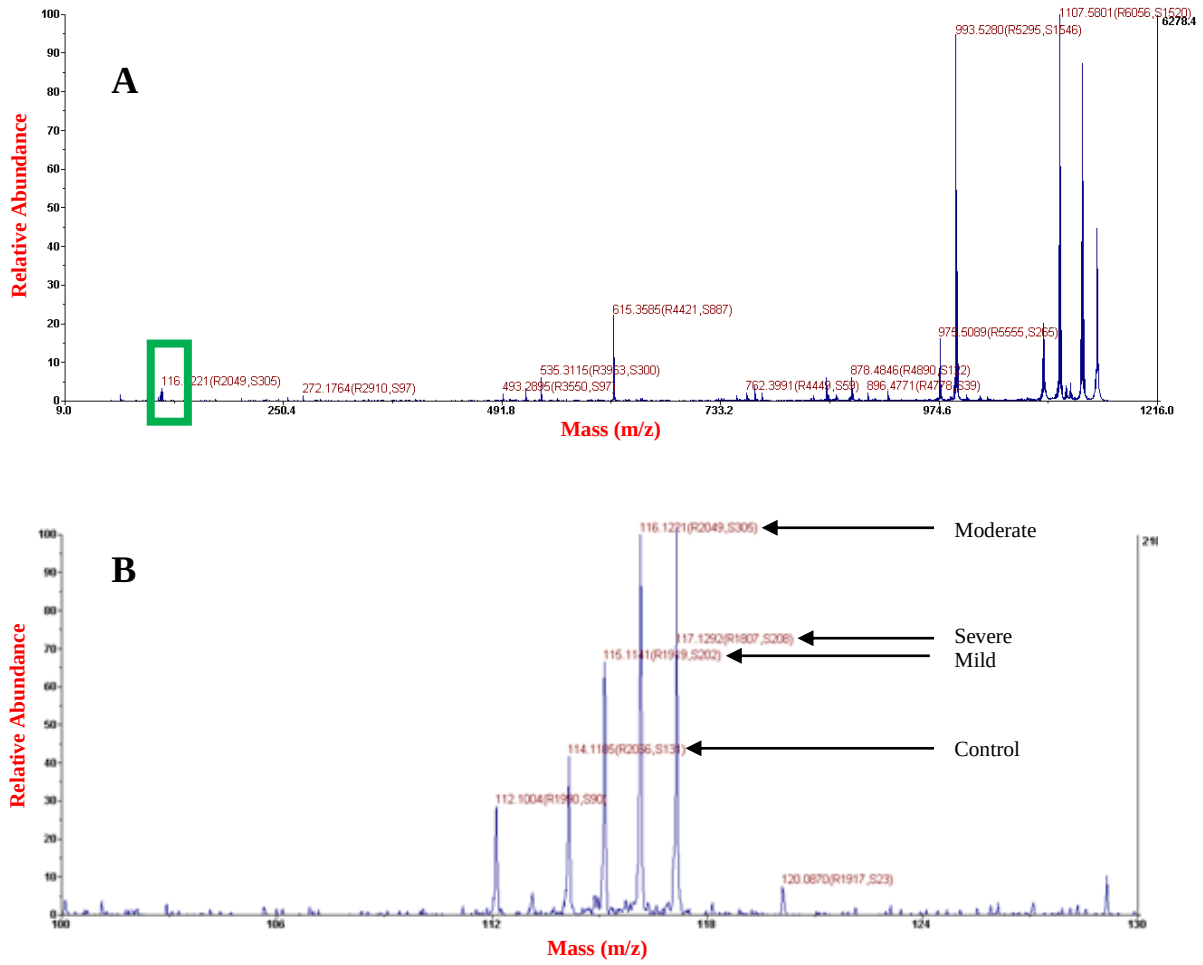

Figure 6.6: MS data acquired for "Lipopolysaccharide binding protein".

Panel A shows the multiple peaks of the protein spectrum of "Lipopolysaccharide-binding protein" as identified by the "Protein Pilot 4.0 Software" using the peptide sequence SFRPFVPR in the "Swiss-Prot" protein database. These peaks were derived from the sera pools of normal control, mild, moderate and severe TBI groups. The green box (bottom left of panel A) shows iTRAQ signals from each of the 4 groups and are reproduced in Panel B as an enlarged image. The 4 peaks in Panel B indicate the levels of "Lipopolysaccharide-binding protein" protein in these groups. Severe TBI (ratio over control= 7.3) had the highest level of the protein, followed by moderate (ratio over control= 6.9) and mild TBI (ratio over control= 5.3). A ratio of ≥1.6 is considered as a significant increase.

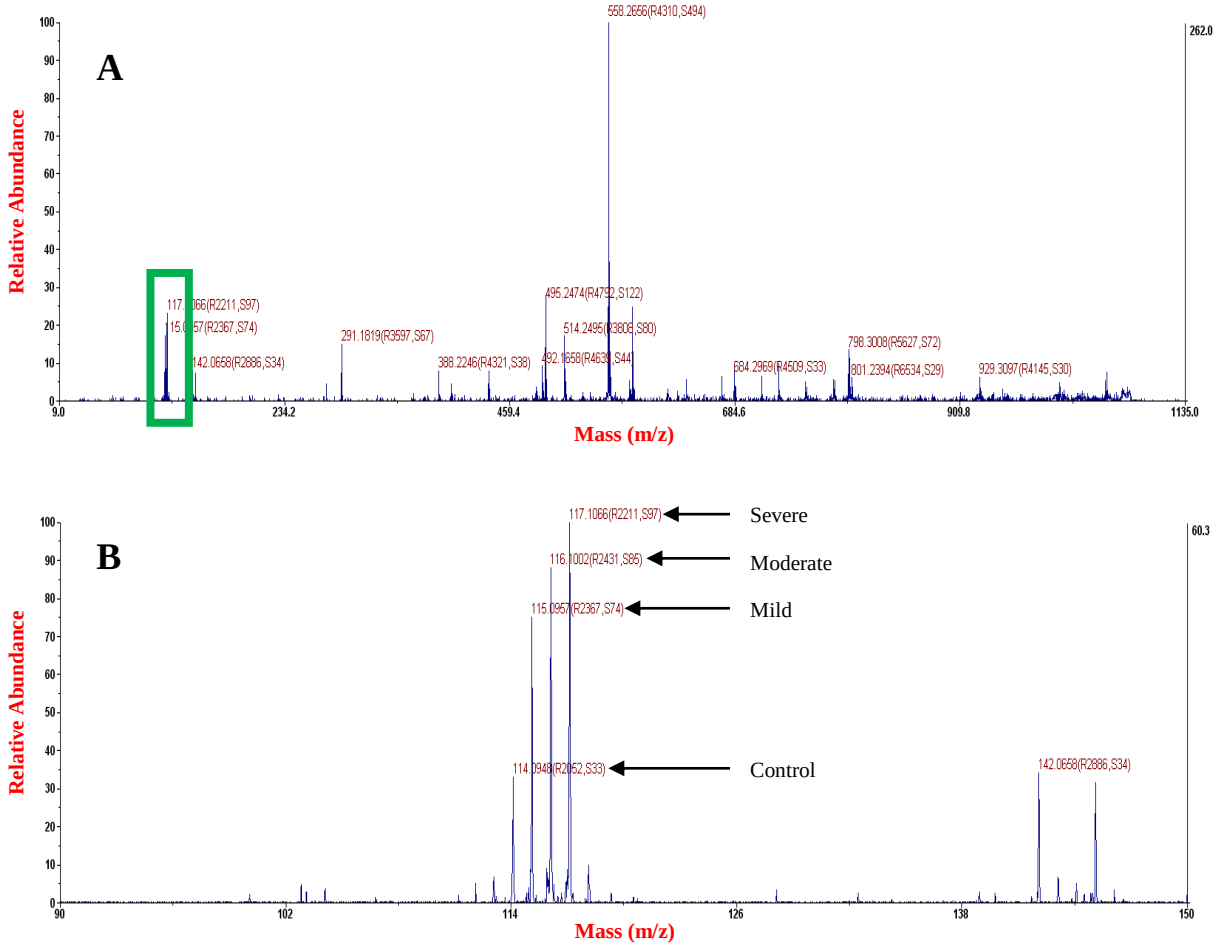

Figure 6.7: MS data acquired for "Fibronectin".

Panel A shows the multiple peaks of the protein spectrum of "Fibronectin" as identified by the "Protein Pilot 4.0 Software" using the peptide sequence MSESGFK in the "Swiss-Prot" protein database. These peaks were derived from the sera pools of normal control, mild, moderate and severe TBI groups. The green box (bottom left of panel A) shows iTRAQ signals from each of the 4 groups and are reproduced in Panel B as an enlarged image. The 4 peaks in Panel B indicate the levels of "Fibronectin" protein in these groups. Severe TBI (ratio over control= 5.2) had the highest level of the protein, followed by moderate (ratio over control= 2.6) and mild TBI (ratio over control= 2.2). A ratio of ≥1.6 is considered as a significant increase.

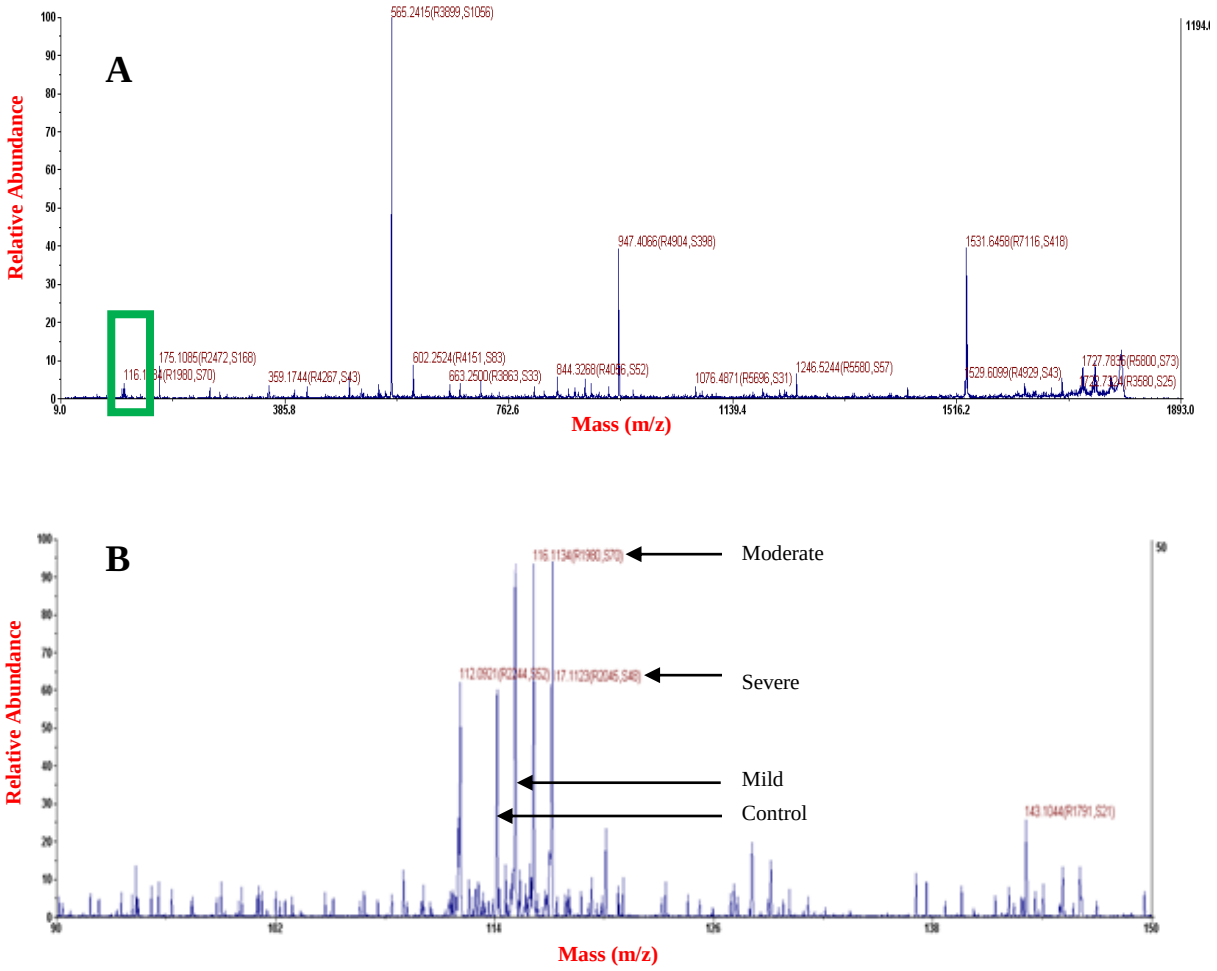

Figure 6.8: MS data acquired for "Vitronectin".

Panel A shows the multiple peaks of the protein spectrum of "Vitronectin" as identified by the "Protein Pilot 4.0 Software" using the peptide sequence DVWGIEGPIDAAFTR in the "Swiss-Prot" protein database. These peaks were derived from the sera pools of normal control, mild, moderate and severe TBI groups. The green box (bottom left of panel A) shows iTRAQ signals from each of the 4 groups and are reproduced in Panel B as an enlarged image. The 4 peaks in Panel B indicate the levels of "Vitronectin" protein in these groups. Mild, moderate and severe TBI had equal levels of protein increase (ratio over control= 1.6). A ratio of ≥1.6 is considered as a significant increase.

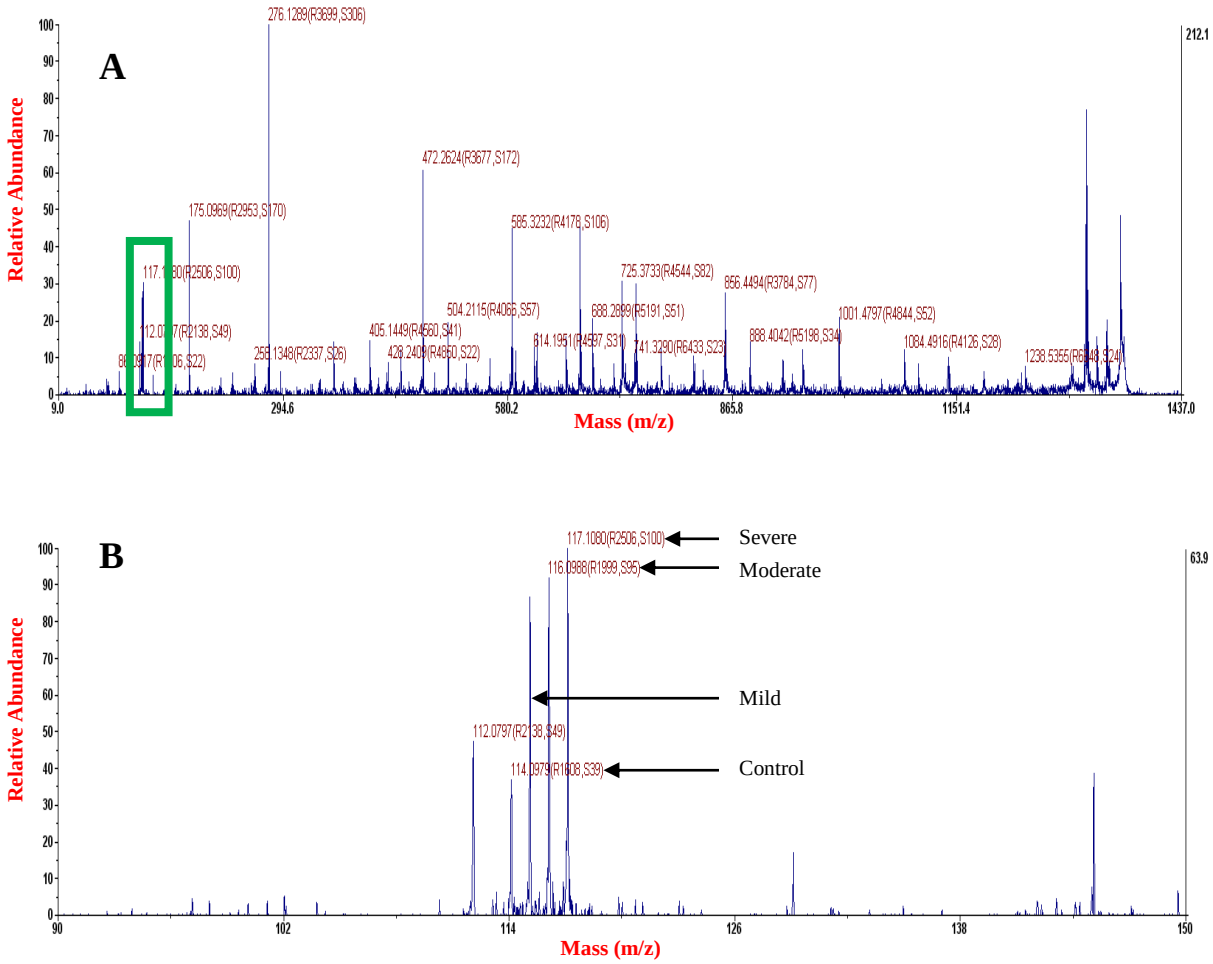

Figure 6.9: MS data acquired for "Alpha-1 antichymotrypsin".

Panel A shows the multiple peaks of the protein spectrum of "Alpha-1 antichymotrypsin" as identified by the "Protein Pilot 4.0 Software" using the peptide sequence ITLLSALVETR in the "Swiss-Prot" protein database. These peaks were derived from the sera pools of normal control, mild, moderate and severe TBI groups. The green box (bottom left of panel A) shows iTRAQ signals from each of the 4 groups and are reproduced in Panel B as an enlarged image. The 4 peaks in Panel B indicate the levels of "Alpha-1 antichymotrypsin" protein in these groups. Severe TBI (ratio over control= 19.0) had the highest level of the protein, followed by moderate (ratio over control= 16.0) and mild TBI (ratio over control= 15.0). A ratio of ≥1.6 is considered as a significant increase.

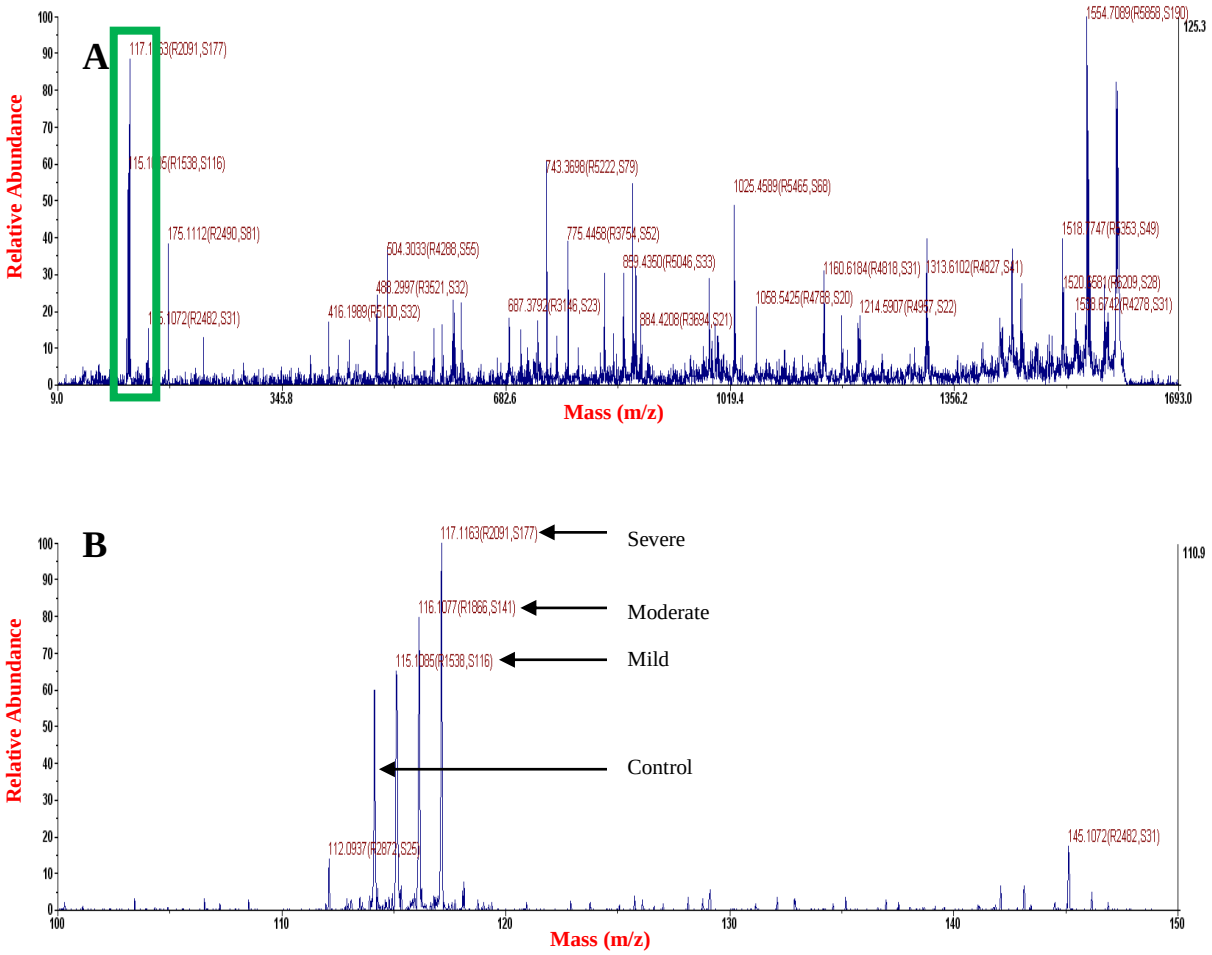

Figure 6.10: MS data acquired for "Apolipoprotein E".

Panel A shows the multiple peaks of the protein spectrum of "Apolipoprotein E" as identified by the "Protein Pilot 4.0 Software" using the peptide sequence AKLEEQAQQIR in the "Swiss-Prot" protein database. These peaks were derived from the sera pools of normal control, mild, moderate and severe TBI groups. The green box (bottom left of panel A) shows iTRAQ signals from each of the 4 groups and are reproduced in Panel B as an enlarged image. The 4 peaks in Panel B indicate the levels of "Apolipoprotein E" protein in these groups. The protein was only increased in severe TBI (ratio over control= 2.3). A ratio of ≥1.6 is considered as a significant increase. The protein levels were not changed significantly in moderate and mild TBI (ratio over control <1.6).

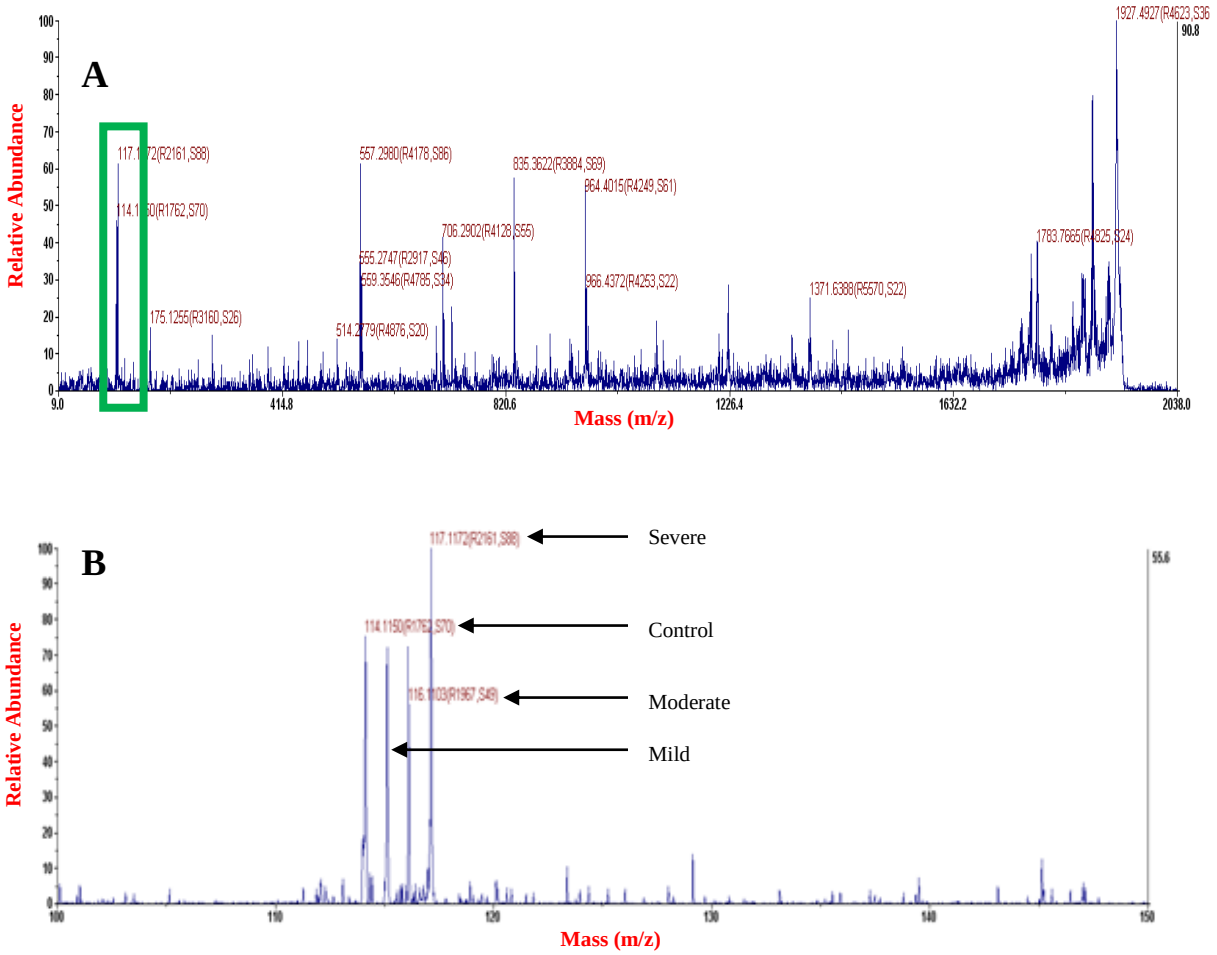

Figure 6.11: MS data acquired for "Zinc alpha-2 glycoprotein".

Panel A shows the multiple peaks of the protein spectrum of "Zinc-alpha-2 glycoprotein" as identified by the "Protein Pilot 4.0 Software" using the peptide sequence AKAYLEEECPATLR in the "Swiss-Prot" protein database. These peaks were derived from the sera pools of normal control, mild, moderate and severe TBI groups. The green box (bottom left of panel A) shows iTRAQ signals from each of the 4 groups and are reproduced in Panel B as an enlarged image. The 4 peaks in Panel B indicate the levels of "Zinc-alpha-2 glycoprotein" protein in these groups. The protein was only increased in severe TBI (ratio over control= 3.2). A ratio of ≥1.6 is considered as a significant increase. The protein levels were not changed significantly in moderate and mild TBI (ratio over control <1.6).

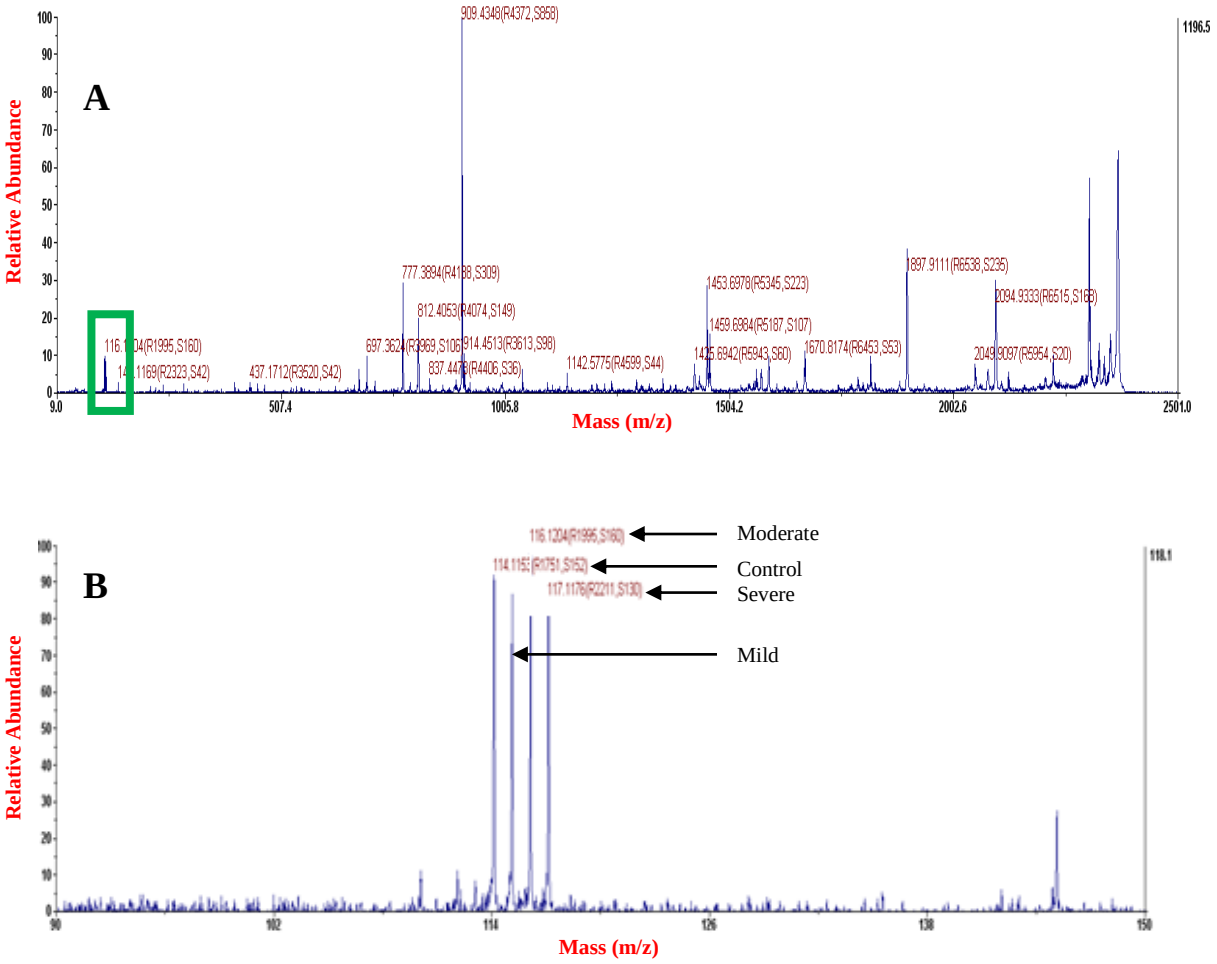

Figure 6.12: MS data acquired for "Gelsolin".

Panel A shows the multiple peaks of the protein spectrum of "Gelsolin" as identified by the "Protein Pilot 4.0 Software" using the peptide sequence VPEARPNSMVVEHPEFLK in the "Swiss-Prot" protein database. These peaks were derived from the sera pools of normal control, mild, moderate and severe TBI groups. The green box (bottom left of panel A) shows iTRAQ signals from each of the 4 groups and are reproduced in Panel B as an enlarged image. The 4 peaks in Panel B indicate the levels of "Gelsolin" protein in these groups. Severe and moderate TBI had the highest levels of the protein decrease (ratio over control= 0.3), followed by mild TBI (ratio over control= 0.4). A ratio of <0.625 is considered as a significant decrease.

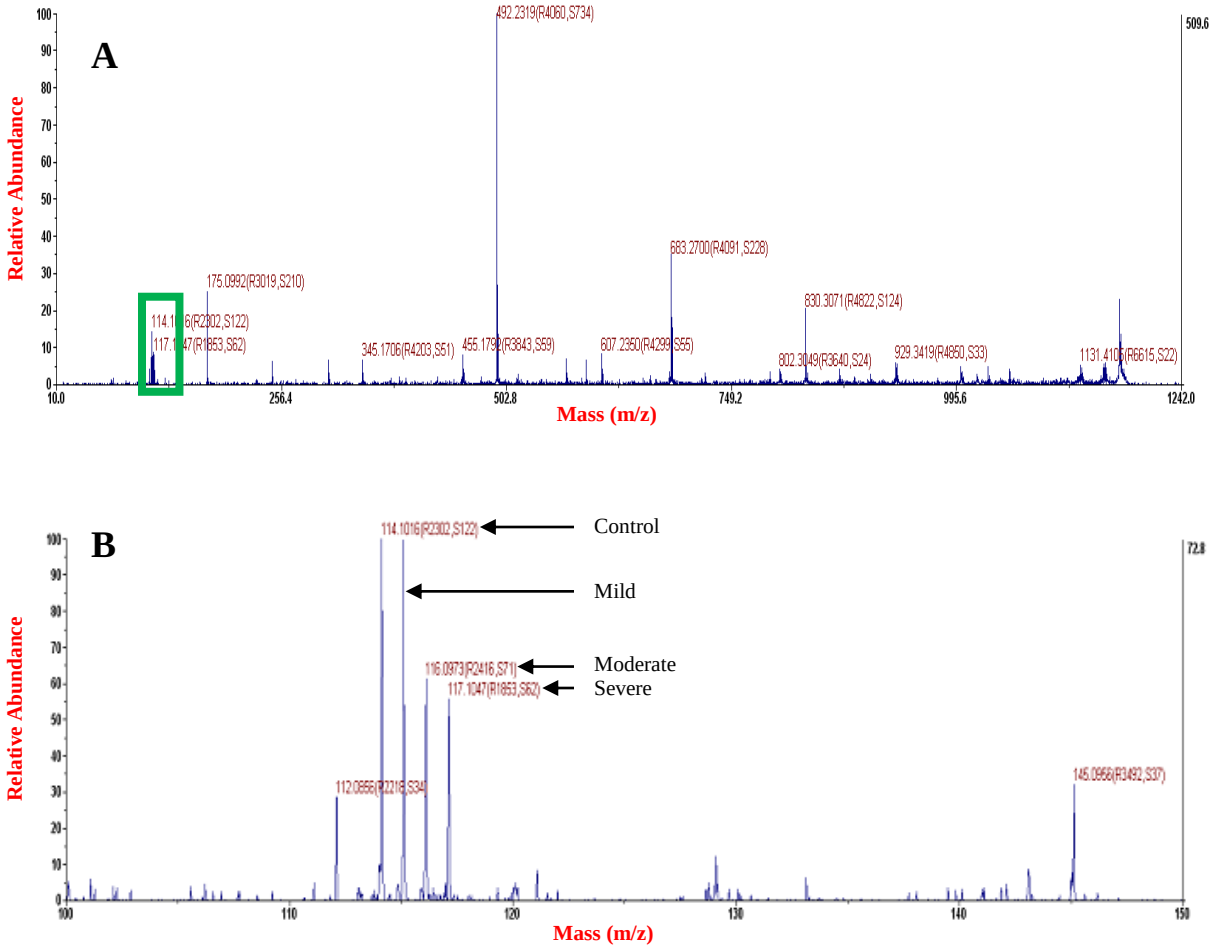

Figure 6.13: MS data acquired for "Kininogen".

Panel A shows the multiple peaks of the protein spectrum of "Kininogen" as identified by the "Protein Pilot 4.0 Software" using the peptide sequence YFIDFVAR in the "Swiss-Prot" protein database. These peaks were derived from the sera pools of normal control, mild, moderate and severe TBI groups. The green box (bottom left of panel A) shows iTRAQ signals from each of the 4 groups and are reproduced in Panel B as an enlarged image. The 4 peaks in Panel B indicate the levels of "Kininogen" protein in these groups. The protein was only decreased in severe and moderate TBI (ratio over control= 0.4). A ratio of <0.625 is considered as a significant decrease. The protein levels was not changed in mild TBI (ratio over control >0.625).

Based on this criteria, 7 of these 11 biomarkers were increased in all the grades of TBI (Table 6.3). Among these 7 markers, except for “Alpha-1 antichymotrypsin”, 4 of these markers (“Leucine-Rich alpha-2 Glycoprotein-1”, “Lipopolysaccharide Binding Protein”, “Fibronectin” and “Vitronectin”) appear to be novel biomarkers for TBI. Two markers, “Serum Amyloid-A” and “C-reactive Protein”, are known general markers of inflammation. The serum biomarker with the highest ratio of increase was “Serum Amyloid-A” (severe TBI/control ratio = 47.9; moderate TBI/control ratio = 35.3 and mild TBI/control ratio = 29.4), and the lowest ratio of increase was “Vitronectin” (with the same ratio of 1.6 for severe, moderate and mild TBI) (Table 6.3). Moreover, except for “Vitronectin”, all the other proteins viz., “Serum Amyloid-A”, “C-reactive Protein”, “Leucine-Rich alpha-2 Glycoprotein-1”, “Lipopolysaccharide Binding Protein”, “Fibronectin”, and “Alpha-1 antichymotrypsin”, showed the most definite gradual increase of proteins from mild, moderate to severe TBI groups (Table 6.3). Two biomarkers, “Apolipoprotein E” and “Zinc alpha-2 glycoprotein” were only increased in severe TBI (Table 6.3). Most of these increased serum biomarkers were secreted proteins except “Fibronectin” and “Apolipoprotein E”. “Fibronectin” is an extracellular matrix structural constituent, whereas “Apolipoprotein E” is a constituent of lipid-protein complexes.

On the other hand, “Gelsolin” was more or less equally decreased in all grades of TBI compared to the controls, whereas “Kininogen” was equally decreased in moderate and severe TBI (Table 6.3). Only “Kininogen” is apparently novel for TBI. “Gelsolin” is a cytoplasmic component, a structural constituent of cytoskeleton as well as a secretory protein. “Kininogen” is a secretory protein.

Table 6.3: Serum protein changes in mild, moderate and severe TBI

Protein name (Accession Number)	Ratio *			Biological Process	Subcellular Location	Molecular Function
	Mild/Control	Moderate/ Control	Severe/ Control			
INCREASED						
Serum Amyloid-A (P0DJI8)	29.4	35.3	47.9	Major acute phase reactant	Secreted	G-protein coupled receptor binding
C-reactive Protein (P02741)	16.7	20.0	32.0	Acute phase response	Secreted	Cholesterol binding, choline binding, low-density lipoprotein particle binding, metal ion binding
Leucine-Rich alpha-2-Glycoprotein-1 (P02750)	21.3	23.8	37.0	Unclassified	Secreted	Unclassified
Lipopolysaccharide-Binding Protein (P18428)	5.3	6.9	7.3	Lipid transport, transport	Secreted	Lipopolysaccharide binding, lipoteichoic acid binding, receptor binding
Fibronectin (P02751)	2.2	2.6	5.2	Acute phase, angiogenesis, Cell adhesion, Cell shape	Extracellular matrix, Secreted	Collagen binding, extracellular matrix structural constituent, heparin binding, peptidase activator activity
Vitronectin (P04004)	1.6	1.6	1.6	Cell adhesion	Secreted	Extracellular matrix binding, heparin binding, polysaccharide binding, scavenger receptor activity
Alpha-1 antichymotrypsin (P01011)	15.0	16.0	19.0	Acute phase response	Secreted	DNA binding, serine-type endopeptidase inhibitor activity

* The protein level is considered increased if the ratio is ≥1.6 and it is considered decreased if the ratio <0.625

Table 6.3, Continued: Serum protein changes in mild, moderate and severe TBI

Protein name	Ratio *			Biological Process	Subcellular Location	Molecular Function
	Mild/Control	Moderate/ Control	Severe/ Control			
Apolipoprotein E (P02649)	Unchanged	Unchanged	2.3	Cholesterol metabolism, Lipid metabolism, Lipid transport, Steroid metabolism, Sterol metabolism, Transport	Chylomicron, HDL, Secreted, VLDL	Antioxidant activity, cholesterol transporter activity, heparin binding, hydroxyapatite binding, lipid transporter activity, lipoprotein particle binding, low-density lipoprotein particle receptor binding, metal chelating activity, phosphatidylcholine-sterol O-acyltransferase activator activity, phospholipid binding, very-low-density lipoprotein particle receptor binding
Zinc alpha-2-glycoprotein (P25311)	Unchanged	Unchanged	3.2	Lipid degradation	Secreted	Antigen binding, peptide antigen binding, protein transmembrane transporter activity, ribonuclease activity
DECREASED						
Gelsolin (P06396)	0.4	0.3	0.3	Cilium biogenesis or degradation	Amyloid, Cytoplasm, Cytoskeleton, Secreted	Calcium ion binding
Kininogen (P01042)	Unchanged	0.4	0.4	Blood coagulation Hemostasis Inflammatory response	Secreted	Cysteine-type endopeptidase inhibitor activity, heparin binding, zinc ion binding

* The protein level is considered increased if the ratio is ≥1.6 and it is considered decreased if the ratio <0.625

We were able to validate by ELISA in a separate cohort of TBI patients all the 11 serum protein biomarkers that were found to be increased/decreased by mass spectrometry. The mean concentrations of these biomarkers and the P values compared to normal controls and femoral fracture controls are shown in Table 6.4. Similar to mass spectrometry results, the ELISA showed that the serum level for "Serum Amyloid-A", "C-reactive Protein", "Leucine-Rich alpha-2 Glycoprotein-1", "Lipopolysaccharide Binding Protein", "Fibronectin", "Vitronectin" and "Alpha-1 antichymotrypsin" were significantly increased (t-test, P value < 0.05) in all grades of TBI. As expected, the ELISA results also showed that the general inflammatory markers, "Serum Amyloid-A" and "C-reactive protein" levels, were significantly increased in femoral fracture controls (t-test, P value < 0.05), and thus, these markers are nonspecific for TBI. On the other hand, "Leucine-Rich alpha-2 Glycoprotein-1", "Lipopolysaccharide Binding Protein", "Fibronectin", "Vitronectin" and "Alpha-1 antichymotrypsin" were not increased in the femoral fracture controls (t-test, P value > 0.05), suggesting that these biomarkers are specific for TBI (Table 6.4).

"Apolipoprotein E" and "Zinc Alpha-2 glycoprotein" were also confirmed to be only significantly increased in severe TBI compared to normal control samples (t-test, P value < 0.05). These 2 markers were not found to be increased in the femoral fracture controls (t-test, P value > 0.05) (Table 6.4). Furthermore, the ELISA results confirmed that serum level of "Gelsolin" was significantly decreased in all grades of TBI and "Kininogen" was significantly decreased only in moderate and severe TBI (t-test, P Value < 0.05). These markers were unchanged in the femoral fracture controls (t-test, P value > 0.05), suggesting that these biomarkers were specific to TBI.

Table 6.4: Validation of Identified Biomarkers by ELISA

Protein	Normal Control	Mild		Moderate		Severe		Femoral Fracture Control	
	Mean Con. (mg/ml)	Mean Con. (mg/ml)	P value	Mean Con. (mg/ml)	P value	Mean Con. (mg/ml)	P value	Mean Con. (mg/ml)	P value
"Serum Amyloid-A"	0.02 ± 0.0032	0.61 ± 0.0012	0.01	0.71 ± 0.0023	0.01	0.96 ± 0.0046	0.01	0.47 ± 0.0071	0.04
"C-reactive Protein"	0.009 ± 0.0001	0.147 ± 0.0001	0.01	0.182 ± 0.0005	0.01	0.312 ± 0.0002	0.01	0.11 ± 0.0061	0.04
"Leucine-Rich alpha-2 Glycoprotein-1"	0.05 ± 0.002	1.08 ± 0.001	0.01	1.17 ± 0.002	0.01	19.2 ± 0.007	0.01	0.05 ± 0.007	P > 0.05
"Lipopolysaccharide Binding Protein"	0.0181 ± 0.0002	0.0962 ± 0.0001	0.04	0.1324 ± 0.0006	0.03	0.142 ± 0.0002	0.03	0.0211 ± 0.0011	P > 0.05
"Fibronectin"	0.00216 ± 0.00003	0.00487 ± 0.00002	0.04	0.00571 ± 0.00002	0.04	0.01254 ± 0.00007	0.03	0.00201 ± 0.00022	P > 0.05
"Vitronectin"	3.0 ± 0.2	4.6 ± 0.12	0.04	4.7 ± 0.18	0.04	4.7 ± 0.27	0.04	2.9 ± 0.4	P > 0.05
"Alpha-1 antichymotrypsin"	0.48 ± 0.01	7.12 ± 0.02	0.01	7.68 ± 0.02	0.01	9.23 ± 0.06	0.01	0.52 ± 0.02	P > 0.05
"Apolipoprotein E"	0.07 ± 0.003	0.075 ± 0.001	> 0.05	0.078 ± 0.003	> 0.05	0.22 ± 0.005	0.04	0.08 ± 0.007	P > 0.05
"Zinc Alpha-2 glycoprotein"	0.6 ± 0.02	0.62 ± 0.009	> 0.05	0.64 ± 0.007	> 0.05	1.97 ± 0.002	0.04	0.6 ± 0.08	P > 0.05
"Gelsolin"	200 ± 12.1	87 ± 4.2	0.03	54 ± 3.7	0.02	52 ± 2.2	0.02	218 ± 12.0	P > 0.05
"Kininogen"	0.055 ± 0.0008	0.055 ± 0.0003	> 0.05	0.029 ± 0.0001	0.02	0.030 ± 0.0006	0.02	0.061 ± 0.0019	P > 0.05

Abbreviation: Concentration (Con), milligram/milliliter (mg/mL); Footnote: (1) Independent t-test were used to compare the concentration of protein from each TBI groups and femoral fracture group with control group; (2) A P-value <0.05 was considered as significant increase or decrease of the protein.

6.5 Discussion

Polytrauma patients are managed by a multidisciplinary trauma team led by the emergency department consultant based on the 'golden hour in shock' principle. Usually a rapid primary survey will be performed to immediately identify and manage life-threatening injuries, followed by a detailed head-to-toe secondary survey in the resuscitation room. Often a tertiary trauma survey will be performed to identify injuries missed after the primary and secondary surveys (Enderson et al., 1990). Accurate interpretation of the clinical signs and mechanisms of injury is key to avoid missing potential life-threatening injuries.

Despite these efforts, it is recognized that many injuries may still escape detection in the hospital (Enderson & Maull, 1991). Confused unconscious patients or patients under the influence of drugs and/or alcohol, often have difficulty giving an accurate clinical history, leading to missed diagnosis of TBI (Mahoney, Biffl, Harrington, & Cioffi, 2003). This situation is aggravated in mild TBI as symptoms are more subtle, and current technologies are unable to reliably detect neural damage in mild TBI (Borg et al., 2004). The timely diagnosis of TBI is important for acute management, counseling of family members, and provision of rehabilitation services.

The present study used the iTRAQ-based mass spectrometer technology to identify changes, if any, in the levels of serum protein markers that may be specific for TBI. These markers may be able to diagnose and grade mild, moderate or severe TBI. We found 7 biomarkers (“Serum Amyloid-A”, “C-reactive Protein”, “Leucine-Rich alpha-2 Glycoprotein-1”, “Lipopolysaccharide Binding Protein”, “Fibronectin”, “Vitronectin” and “Alpha-1 antichymotrypsin”) to be increased in all grades of TBI (Table 6.3). In addition, except perhaps for “Vitronectin”, these proteins show a definite and gradual increase from mild, moderate to severe TBI groups.

Two of these proteins ("Serum Amyloid-A" and "C-reactive Protein") are markers for general injury and inflammation. Five biomarkers ("Leucine-Rich alpha-2 Glycoprotein-1", "Lipopolysaccharide Binding Protein", "Fibronectin", "Vitronectin" and "Alpha-1 antichymotrypsin") appear to be specific for TBI. Except for "Alpha-1 antichymotrypsin", 4 of these appear to be novel biomarkers for TBI.

"Apolipoprotein E" and "Zinc Alpha-2 glycoprotein" were found to be increased only in severe TBI, but had been previously reported to be increased in TBI. "Gelsolin", was found to be decreased in all grades of TBI, whereas, "Kininogen" was decreased in moderate and severe TBI. Only "Kininogen" appears to be novel for TBI.

Validation with ELISA with an entirely different cohort of patients (normal control, femoral fracture control, mild, moderate, severe TBI groups) further confirmed that the serum level for "Serum Amyloid-A", "C-reactive Protein", "Leucine-Rich alpha-2 Glycoprotein-1", "Lipopolysaccharide Binding Protein", "Fibronectin", "Vitronectin" and "Alpha-1 antichymotrypsin" were increased in all grades of TBI. As expected, "Serum Amyloid-A" and "C-reactive protein" were confirmed as general inflammatory markers, as these proteins were also increased in the femoral fracture controls. Similarly, "Apolipoprotein E" and "Zinc Alpha-2 glycoprotein" were confirmed by ELISA to be increased only in severe TBI, and serum level to be decreased for "Gelsolin" in all TBI grades and "Kininogen" in moderate/severe TBI.

If further validated in larger cohort studies, some or all of these protein biomarkers may aid in the detection of TBI, including mild TBI, especially in patients with polytrauma. Based on our findings, it may be possible that a serum sample taken at 24 hours of the trauma or even before 24 hours, could supplement the diagnosis of TBI using a panel of the 5 specific biomarkers that are increased and 1 specific biomarker that is decreased in all grades of TBI (Table 6.5). With the addition of "Kininogen",

“Apolipoprotein E” and “Zinc Alpha-2 glycoprotein” it may be possible to grade TBI as mild, moderate and severe (Table 6.5).

Table 6.5: Panel of specific biomarkers for diagnosis and grading of TBI

	Specific TBI Biomarkers			
	"Leucine-Rich alpha-2 Glycoprotein-1" "Lipopolysaccharide Binding Protein" "Fibronectin" "Vitronectin" "Alpha-1 antichymotrypsin"	"Gelsolin"	"Kininogen"	"Apolipoprotein E" "Zinc Alpha-2 glycoprotein"
Mild TBI	↑	↓	↔	↔
Moderate TBI	↑	↓	↓	↔
Severe TBI	↑	↓	↓	↑

Symbols: ↑ (increased level in the serum), ↓ (decreased level in serum) and ↔ (unchanged level in the serum)

"Serum Amyloid-A", as a general injury/inflammatory protein marker, has been previously reported to show dramatic increases of up to a mean plasma level of 0.764 g/l after TBI (Saile et al., 1990). However, in large groups of patients with a variety of disorders, rapid production and exceptionally wide dynamic range of the "Serum Amyloid-A" response has been observed thus making it a rather non-specific marker for TBI (Lowe, 2001; Malle & De Beer, 1996). "C-reactive protein" has been reported to be rapidly elevated after brain trauma and are robust indicators of injury at very acute time points (Hergenroeder et al., 2008; Lin, Howng, Hu, & Huang, 1992). However, "C-reactive protein" is also elevated in infections or trauma unrelated to TBI (Hergenroeder et al., 2008). Moreover, "C-reactive protein" levels were increased corresponding to the degree of intra-operative surgical insult (Al-Jabi & El-Shawarby, 2010). Similar to these studies we found that, "Serum Amyloid-A" and "C-reactive protein" were gradually increased in all grades of TBI, as well as significantly increased in femoral fracture controls compared to normal controls. Hence, these proteins are not specific markers for TBI and are unsuitable to be used in the setting of polytrauma.

"Leucine-Rich alpha-2 Glycoprotein-1" is a highly conserved member of the leucine-rich repeat family of proteins involved in protein-protein interaction, signaling and cell adhesion. It is distributed throughout the entire brain, with an especially high expression in the deep cerebral cortex. The expression was observed in resident astrocytes, as well as in the capillary onto which astrocytic processes grow and adhere (Nakajima et al., 2012). "Leucine-Rich alpha-2 Glycoprotein-1" was found to be a marker of interest for idiopathic normal pressure hydrocephalus as it levels in cerebrospinal fluid (together with tau protein and positive cerebrospinal fluid Tap Test) can reliably predict shunting outcome in idiopathic normal pressure hydrocephalus patients (Nakajima, Arai, & Miyajima, 2010; Nakajima et al., 2012; Nakajima et al., 2011). We found this apparently novel TBI biomarker to be significantly increased gradually in all grades of

TBI. The significance of raised "Leucine-Rich alpha-2 Glycoprotein-1" after TBI is uncertain and requires further research to elucidate its role in brain injury.

"Lipopolysaccharide Binding Protein" is a glycosylated 60 kDa serum protein that is primarily produced in the liver (Ramadori, Meyer zum Buschenfelde, Tobias, Mathison, & Ulevitch, 1990). It has been recognized as a marker of overall inflammation in systemic inflammatory response syndrome, a subset of cytokine storm, in which there is abnormal regulation of various cytokines arising from an infectious or a noninfectious insult (Davies & Hagen, 1997). It has been shown that "Lipopolysaccharide Binding Protein" concentrations in non-TBI trauma patients on admission were significantly greater in non- survivors than in survivors. The same study also showed that among the survivors, older patients (age ≥65 years) had higher levels of "Lipopolysaccharide Binding Protein" than younger patients on admission (Cunningham et al., 2006). The authors further speculate that, the mean "Lipopolysaccharide Binding Protein" concentrations on admission and at 24 hour after admission were similar to the concentrations previously reported in septic patients (Sakr, Burgett, Nacul, Reinhart, & Brunkhorst, 2008). However, none of the trauma patients in their study had infection at the time of hospital admission. The finding that plasma "Lipopolysaccharide Binding Protein" concentrations are increased to the same degree as in patients with severe sepsis and septic shock is interesting, but the underlying differences in the mechanisms are not known. "Lipopolysaccharide Binding Protein" was increased gradually in all the grades of TBI in our study but its role in TBI is not known.

"Fibronectin", a high-molecular weight glycoprotein of the extracellular matrix, plays a major role in cell adhesion, growth, migration and differentiation. As it binds extracellular matrix components such as collagen, fibrin, and heparan sulfate proteoglycans, it is important for processes such as wound healing and embryonic development. Egan et al. (1991) detected "fibronectin" in neutropils and non-neuronal

cells adjacent to wounds in rat cerebral cortex (R. A. Egan & K Vijayan, 1991). Both expression of "fibronectin" and increased mRNA was found after TBI in the rat model (Chen et al., 2001). Plasma "fibronectin" deficient mouse showed increased neuronal apoptosis and larger areas of infarction following transient focal cerebral ischemia (Sakai et al., 2001). These mice also showed worse performances on motor and cognitive tasks and had significantly less phagocytic cells in the injured cortex compared to mice with normal plasma "fibronectin" levels. Tate et al. (2002) further showed that intravenous injections of "fibronectin" prior to the injury restored the neural deficits seen in the plasma "fibronectin" deficient mice demonstrating that plasma "fibronectin" may be neuroprotective (Tate et al., 2002). The increased "fibronectin" found in our study may inhibit neuronal apoptosis. However, further studies are required to better understand the mechanisms of neuroprotection, if any.

"Vitronectin" is a 75 kDa glycoprotein that consists of 459 amino acid residues involved with cell adhesion and spread. It is found in serum and the extracellular matrix. In primary rat neuron cultures which were treated with lipoteichoic acid (used to induce/mimic secondary TBI inflammatory reaction), blocking microglial "vitronectin" receptors efficiently prevented neuronal death caused by microglial phagocytosis (Neher et al., 2011). Another study found that TNF-α induced microglial phagocytosis of neurons can be blocked by inhibiting microglial "vitronectin" receptors (Neniskyte, Vilalta, & Brown, 2014). This has raised the concern that "vitronectin" may have neurotoxic effect on neurons by increasing microglial activity. "Vitronectin" itself has been immunolocalized to senile plaques and neurofibrillary tangles in Alzheimer entorhinal cortex, and the senile plaques had microglial cores that were strongly positive for the "vitronectin" receptor. The high levels of "vitronectin" receptor on reactive microglia in areas containing extracellular "vitronectin" suggest the possibility that "vitronectin" is serving an opsonizing function for microglial phagocytosis (Akiyama, Kawamata,

Dedhar, & McGeer, 1991). It is possible that increased "Vitronectin" in TBI, may enhance phagocytic action against neurons that may have a deleterious effect on post-TBI recovery. Further studies are required to understand the mechanisms involving "Vitronectin" in TBI.

"Alpha-1 antichymotrypsin" is an alpha globulin glycoprotein and a member of the serpin superfamily. It inhibits the activity of proteases, e.g. cathepsin G of neutrophils, and chymases of mast cells, by cleaving them into a different shape or conformation. "Alpha-1 antichymotrypsin" was found to be expressed by reactive astrocytes around cerebral wound margins between 18 hours to 13 days after neural lesions in mouse and are believed to assist in early wound repair (Abraham, 1992; Abraham, Kanemaru, & Mucke, 1993). "Alpha-1 antichymotrypsin" has also been identified as a major constituent of the neurofibrillary plaques associated with Alzheimer's disease, and in vitro studies have shown that it enhances the rate of amyloid-fibril formation (Padmanabhan, Levy, Dickson, & Potter, 2006). Furthermore, a study of biomarkers in severe TBI using similar iTRAQ-based mass spectrometry also found "Alpha-1 antichymotrypsin" to be increased. However, this study did not investigate the level of this biomarker in mild and moderate TBI (Hergenroeder et al., 2008). The significance of this biomarker in TBI requires further research to elucidate its role in TBI.

"Apolipoprotein E" is a 299 amino acid protein with a relative molecular mass of 34 KDa. It is the major apolipoprotein in human cerebrospinal fluid and is synthesized by astrocytes (Mahley, 1988; Mahley et al., 1984). It exist as small spherical, discoidal lipoproteins, often packed together with cholesterol and phospholipid to form lipid-protein complexes. These complexes bind to "Apolipoprotein E" receptors on the surfaces of nerve cells and are internalised into the cell. In our study, serum "Apolipoprotein E" was found to be significantly increased only in severe TBI. Interestingly, as long as two decades ago, "Apolipoprotein E" had been proposed as a marker of severe TBI, its levels

reaching three times the normal value 10 days after severe TBI (Kabbaj et al., 1989). It has been suggested that "Apolipoprotein E" may play a role in neuronal homeostasis (Laws et al., 2003), particularly, the mobilization of cholesterol in the CNS where it is required for neuronal plasticity (Moestrup et al., 1992; Rebeck et al., 1993). "Apolipoprotein E" is also postulated to be involved with neuronal repair by mediating the recycle of damaged cell membranes (Laws et al., 2003).

"Zinc Alpha-2 glycoprotein" is a 40-kDa single-chain polypeptide (Burgi & Schmid, 1961) is involved preferentially in depletion of fatty acids from adipose tissues, thus subsequently named as a lipid-mobilizing factor (Bao et al., 2005). In the cerebrospinal fluid, an increased level of "Zinc Alpha-2 glycoprotein" had been previously proposed as a marker for Parkinson's Disease (Yin, Lee, Cho, & Suk, 2009; Zhao, Xiao, Pu, & Zhong, 2010) but the underlying mechanism for the increase is unknown. Similar to our findings, "Zinc Alpha-2 glycoprotein" was also found to be increased in severe TBI patients using the same iTRAQ- based mass spectrometry approach (Hergenroeder et al., 2008). The significance of its elevation in severe TBI but not in mild or moderate TBI needs further investigation.

"Gelsolin" is an 82-kD actin-binding protein that regulates the assembly and disassembly of actin filaments. This biomarker was found to be reduced after TBI in the current study. Other studies also showed that plasma "gelsolin" level in TBI patients decreased after TBI compared to healthy controls (Jin, Li, Qiu, Ling, & Bai, 2012; Xu, Liu, Dong, Yang, & Fan, 2012). Xu et al., (2012) further reported that "gelsolin" level decreased during the 6-hour period immediately after TBI, was at the nadir in 24 hours, increased gradually thereafter, and was substantially lower than that in healthy controls during the 7-day period (Xu et al., 2012). Similar iTRAQ-based mass spectrometry also showed that "gelsolin" levels were decreased in severe TBI (Hergenroeder et al., 2008).

However, the role of "gelsolin" in TBI and why there is a decrease in its levels remains uncertain and requires further research.

Kininogen is a 299 amino acid protein, which upon cleavage by human plasma kallikrein, or factor XIIa it releases and activates kinin (Colman & Muller-Esterl, 1988) in the CNS. Kinins are pro- inflammatory mediators involved in pain, edema, brain blood barrier disruption and are associated with neurodegenerative disorders (Guevara-Lora, 2012). In our study, "kininogen" was found to be decreased only in moderate and severe TBI suggesting that it may be able to differentiate moderate/severe TBI from mild TBI. We speculate that its decrease in moderate and severe TBI may be a mechanism that could help to protect neurons. "Kininogen" has never been studied in human serum as a marker for TBI. However, in a study by Ellis et al., injury in the right cerebral cortex of rats showed the "kininogen" content in the right hemisphere was significantly elevated within one hour after injury and continued to rise until 15 hours after injury. The reason for the contrast between increased "Kininogen" level in cerebral cortex of rat and decreased "Kininogen" in human serum is uncertain and requires further research to elucidate the role of this protein in brain injury.

Except for "Serum Amyloid-A" and "C-reactive Protein", our study did not confirm the usefulness of other putative serum biomarkers that were previously shown to be raised in TBI, including Interleukin 6, Interleukin 8, Interleukin 10, Interleukin 12p70, Tumor Necrosis Factor α, Glial Fibrillary Acidic Protein, Creatine Kinase Brain Isoenzyme and Cleaved tau (Table 6.1). In fact, these proteins were not detected in our study samples so they were not studied further. Similar to the findings in our study, these serum biomarkers were also not detected in severe TBI using the iTRAQ-based mass spectrometry approach (Hergenroeder et al., 2008). We are not sure why these proteins were not detected by iTRAQ-based mass spectrometry. One possible reason may be that generally mass spectrometry is unable to identify all the proteins in very complex

samples, such as pooled sera (Bantscheff, Lemeer, Savitski, & Kuster, 2012; Schulze & Usadel, 2010). In mass spectrometry, the presence of 4 to 5 peptides are usually adequate to accurately identify a known protein. However, as the number of proteins contained within a sample increases, the required number of peptides sequences to positively identify a specific protein also increases. It is possible that these proteins could not be identified in our samples because of this. However, it cannot be over emphasized that all the proteins of interests detected by iTRAQ-based mass spectrometry were confirmed by ELISA.

Using a targeted ELISA method, "Interleukin 6" (Chiaretti et al., 2008; Winter et al., 2004), "Interleukin 8" (Buttram et al., 2007), "Interleukin 10" (Bell et al., 1997; Csuka et al., 1999), "Interleukin 12p70" (Buttram et al., 2007), "Tumor Necrosis Factor α" (Crespo et al., 2007), Glial Fibrillary Acidic Protein (Missler et al., 1999; Nylen et al., 2006; Pelinka, Kroepfl, et al., 2004; Vos et al., 2004), Creatine Kinase Brain Isoenzyme (Carr, Masullo, Brown, & Lewis, 2009; Schwartz et al., 1989; Skogseid et al., 1992) and Cleaved tau (Guzel, Karasalihoglu, Aylanc, Temizoz, & Hicdonmez, 2010; Kavalci et al., 2007; Liliang et al., 2010) had been reported in literature as possible TBI markers. Unfortunately, Interleukin 6, Interleukin 8, Interleukin 10, Interleukin 12p70 and Tumor Necrosis Factor α, being cytokines may also be increased in other systemic injuries/conditions such as infection, extracranial trauma or organ failures (Chiaretti et al., 2008; Chiaretti et al., 2005; Csuka et al., 1999; Hergenroeder et al., 2008; Winter et al., 2004). Among the 3 brain-specific proteins, viz., "Glial Fibrillary Acidic Protein", "Creatine Kinase Brain Isoenzyme" and "Cleaved tau", "Glial Fibrillary Acidic Protein" was suggested to be the most promising marker for TBI (Missler et al., 1999; Vos et al., 2004). However, its increase in serum of patients with mild TBI is inconsistent, and might be below the detectable limit (Zemlan et al., 2002).

The serum protein markers identified in this study should be confirmed as specific and/or sensitive for the detection and grading of TBI by validating its usefulness in larger cohorts of TBI patients. Moreover, the alterations of protein levels need to be evaluated in other disease control groups such as infections, autoimmune diseases and cancers. As protein levels changes over time and also may be altered by treatment, studies on how these parameters impact on the protein levels may be important. Studies of autopsy brain tissues may also be needed further understand the role of these proteins in TBI. Neurotoxic or neuroprotective effects, if any, of these protein markers need to be established by developing cell/tissue culture or animal models.

6.6 Conclusion

Alterations in levels of serum proteins within 24 hours in patients with mild, moderate and severe TBI may be useful as an adjunct to detect and even grade TBI, especially in the polytrauma setting. These biomarkers allow a quantitative diagnostic, even for patients who cannot be evaluated by GCS due to intubation, ventilation and/or sedation. For this purpose, we propose a panel of serum proteins that includes “Leucine rich alpha-2 glycoprotein-1”, “Lipopolysaccharide binding protein”, “Fibronectin”, “Vitronectin” and “Alpha-1 antichymotrypsin” and “gelsolin” to diagnose TBI. In addition to these biomarkers, “Kininogen”, “Apolipoprotein E” and “Zinc Alpha-2 glycoprotein” may further able to grade TBI into mild, moderate and severe. Especially in mild TBI, as the patients’ GCS scores are normal, and neuroimaging investigations such as the CT scan or brain MRI may not show evidence of injury.

CHAPTER 7

CONCLUSION AND RECOMMENDATION

We have found that possession of either the *APOE* ε4 allele or *COMT* AA genotype or *CNTF* A allele was independently associated with poor outcome at 6 months after TBI. Combined with the GCS these parameters were even better predictors for unfavorable outcome at 6 month compared with GCS alone. All these 3 SNPs should be further investigated and confirmed as prognostic indicators in larger cohort studies. We did not find any association between SNPs in the *APOE* promoter region, *DRD2* gene, *DRD3* gene, *BDNF* gene and *GDNF* gene with outcome after TBI.

It is important to note that the contribution of SNPs to outcome may be modest as outcome after TBI is a complex interaction of the type of brain injury, effects of injury to other areas, post-injury treatment and various psychosocial factors. Genome Wide Association Studies (GWAS) are particularly useful in identifying groups of genetic variants or regions of interest within a specific gene that can be further analyzed to better characterize pathophysiologic pathways. These studies are exploratory in nature and require screening of millions of SNPs for an association with an outcome of interest. A major limitation for GWAS is the large number (> 100,000 cases) of samples required to attain robust statistical significance. Hence, a multi-center study with much larger sample size is needed to confirm our results. In this study we only utilized GOS to measure the functional outcome after TBI but other parameters such as neurocognitive and neuropsychological outcomes may also be studied.

Altogether we found 9 serum protein biomarkers viz., “Serum Amyloid-A”, “C-reactive Protein”, “Leucine-Rich alpha-2 Glycoprotein-1”, “Lipopolysaccharide Binding Protein”, “Fibronectin”, “Vitronectin” and “Alpha-1 antichymotrypsin”, to be increased in mild, moderate and severe TBI, while a further 2 “Apolipoprotein E” and “Zinc Alpha-2

glycoprotein" were only increased in severe TBI. However, "Serum Amyloid-A" and "C-reactive Protein" are considered as general injury and inflammatory markers whereas the other 7 may be more specific. Some of the specific protein markers that were increased in mild/moderate/severe TBI may aid in the detection of mild TBI, which is often missed in the initial diagnostic assessment. Decreased levels of "Gelsolin" in mild/moderate/severe TBI and "Kininogen" in moderate/severe TBI may be specific to TBI. Our findings suggest that a 24 hour post-trauma serum protein profile of some or all of these biomarkers may be of the trauma useful as an adjunct to confirm and/or help grade TBI to complement the traditional use of the GCS score.

Further investigations are needed to assess the specificity and sensitivity of the proposed markers in larger cohorts, in particular the proteins that appear to be novel to TBI ("Leucine- Rich alpha-2 Glycoprotein-1", "Lipopolysaccharide Binding Protein", "Fibronectin", "Vitronectin" and "Kininogen"). Most importantly, changes in the serum levels of these markers over time need also to be established. Changes in response to treatment, if any, may help elucidate the role of these markers on outcome after TBI. Histopathological studies in autopsy brain tissue may be useful to map the distribution of these proteins in the CNS. Finally, neurotoxic or neuroprotective effect of these protein markers need to be established by developing cell culture model, tissue culture model or animal model.

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SUPPLEMENTARY

I. LIST OF PUBLICATIONS AND PAPER PRESENTED

A. PUBLICATIONS

Anada, R. P., Ganesan, D., Ramahsamay, N., & Wong, K. T. (2012). A prevalence study of single nucleotide polymorphisms in the promoter of the apolipoprotein E gene in different ethnic groups in Malaysia. Neurology Asia, 17(4), 341-346.

Veeramuthu, V., Pancharatnam, D., Poovindran, A. R., Musthapha, N. A., Thong, W. K., Mazlan, M., . . . Ganesan, D. (2014). Cognitive impairments in mild traumatic brain injury and genetic polymorphism of apolipoprotein E: A preliminary study in a Level I trauma center. Neurology Asia, 19(1).

B. PAPER PRESENTED

Polymorphism in the APOE gene and promoter in the functional outcome of traumatic brain injury in the Malaysian population, 16th BIOLOGICAL SCIENCES GRADUATE CONGRESS, Singapore, Dec 2011

Significant association of APOE- ε4 allele and unfavorable outcome after traumatic brain injury: Meta- analysis, ASIA PACIFIC CLINICAL EPIDEMIOLOGY AND EVIDENCE BASED MEDICINE CONFERENCE, Kuala Lumpur, July 2012

II. APPENDICES

APPENDIX A: REAGENTS PREPARATION FOR DNA EXTRACTION AND GEL ELECTROPHORESIS

1. **20X Standard Saline Citrate buffer**

   Materials:

   175.3g of NaCl

   88.2g of sodium citrate

   Method:

   The chemicals dissolved in 800 mL of ultrapure H_2O. The pH was adjusted to 7.0 with a few drops of 1M HCl. The final volume adjusted to 1L with additional ultrapure H_2O. The reagent sterilized by autoclaving.

2. **1X Standard Saline Citrate buffer**

   Materials:

   20X SSC Buffer

   Method:

   Dissolve 25 mL of 20X Standard Saline Citrate buffer in 475 mL of ultrapure H_2O to prepare 500 mL of 1X Standard Saline Citrate buffer. The reagent sterilized by autoclaving.

3. **Proteinase K**

Materials:

20 mg of Proteinase K

Method:

Dissolve 20 mg of Proteinase K in 1 mL of ultrapure H_2O. Do not autoclave as this will denature the enzyme. Aliquots were stored at -20ºC.

4. **10% Sodium Dodecyl Sulphate Buffer**

Materials:

10 g Sodium Dodecyl Sulphate

Method:

Dissolve 10 g Sodium Dodecyl Sulphate in 100 mL ultrapure H_2O. Do not autoclave.

5. **2M Nacl**

Materials:

117 g NaCl

Method:

Dissolve 117 g of NaCl in 1 L of ultrapure H_2O. The reagent sterilized by autoclaving.

6. **10:1 Tris-EDTA buffer**

Materials:

10 mL 1 M Tris-HCl, pH 7.6

2 ml 0.5 M EDTA

Method:

Dissolve the solution in ultrapure H_2O to 1L. The reagent sterilized by autoclaving.

7. **1 M Tris- HCl**

Materials:

121.1 g Tris base

Method:

121.1 g of Tris base dissolved in 800 ml of ultrapure H_2O. The pH adjusted to 7.6 with concentrated HCl and ultrapure H_2O added to 1 L. The reagent sterilized by autoclaving.

8. **0.5 M EDTA**

Materials:

186.1 g Na_2EDTA

ddH2O to 500 millilitre (ml)

Total volume 1000 millilitre (ml)

Note:

Method:

Na_2EDTA dissolved in 400 mL ultrapure H_2O and pH adjusted to 8.0 with 10 N NaOH. Final volume adjusted to 1 L with ultrapure H_2O. The reagent sterilized by autoclaving.

9. **50X Tris-acetate-EDTA buffer (TAE)**

Materials:

240 g Tris base

57.1 mL Glacial acetic acid

100 mL 0.5 M EDTA

Method:

The materials were dissolved to 1L of ultrapure H_2O. The reagent sterilized by autoclaving.

10. **1.5% Agarose Gel**

Materials:

0.45 g Agarose Powder

30 ml of TAE buffer

1 µl of Ethidium Bromide Solution

Method:

0.45g of Agarose powder dissolved in 3o mL of TAE buffer by heating for 1 min in microwove oven. The solution let to cooldown and 1 ul of Ethidium Bromide solution were added. The mixture poured on gel casting tray with gel comb.

APPENDIX B: COMPLETE PROTOCOL FOR DNA EXTRACTION FROM FROZEN BLOOD SAMPLES

1. Blood samples typically were obtained as 1 mL of whole blood stored in EDTA vacutainer tubes frozen at -80˚C.
2. Frozen samples were thawed, and to each 1 mL sample, 0.8 mL 1X SSC buffer added, and mixed. The mixture centrifuged for 20 minute at 13,000 rpm in a microcentrifuge.
3. 1 mL of the supernatant removed and discarded into disinfectant.
4. 1 mL of 1X SSC buffer added, vortexed, centrifuged as above and all of the supernatant removed. The washing step repeated until the pellet appears white.
5. 375 µl of 0.2M NaCL were added to each pellet and vortexed briefly. Then add 25 µl of 10% SDS and 5 µl of proteinase K (20mg/ml H2O) were added, vortexed briefly and incubated for 1 hour at 55˚C.
6. 200 µl phenol/chloroform/isoamyl alcohol were added and mixed gently for 30 seconds. The sample centrifuged for 10 minutes at 13,000 rpm in a microcentrifuge tube.
7. The aqueous layer were carefully removed to a new 1.5 mL microcentrifuge tube.
8. 1 mL of cold 100% ethanol added, mixed, and incubated for 15 minutes at -20˚C.
9. The mixture centrifuged for 2 minutes at 12,000 rpm in a microcentrifuge. The supernatant decanted and drained.
10. 180 ul 10:1 TE buffer were added, vortexed, and incubated at 55˚C for 10 minutes.
11. 20 µl 2M NaCl added and mixed.
12. 500 µl of cold 100% ethanol were added, mixed, and centrifuged for 1 minute at 12,000 rpm in a microcentrifuge.
13. The supernatant decanted and the pellet rinsed with 1 mL of 80% ethanol. Mixture centrifuged for 1 minute at 12,000 rpm in a microcentrifuge.

14. The supernatant decanted and the pellet dried in a clean drying oven overnite at 37˚C (or until dry).
15. The pellet resuspended by adding 200 µl of 10:1 TE buffer.
16. The mixture incubated overnight at 55˚C, vortexing periodically to dissolve the genomic DNA.
17. The samples store at -20˚C.

APPENDIX C: REGION OF *APOE* GENE AND PRIMERS

APOE

Sense Primer	5'-CTG GAG GAA CAA CTG ACC CCG GTG- 3'
Anti- Sense Primer	5'-CAG GCG CTC GCG GAT GGC GCT GAG-3'

Region of APOE gene covered

5'-ctggaggaacaactgaccccggtggcggaggagacgcgggcacggctgtccaaggagctgcaggcggcgca ggcccggctgggcgcggacatggaggacgtgt/cgc[112]ggccgcctggtgcagtaccgcggcgaggtgcaggccat gctcggccagagcaccgaggagctgcgggtgcgcctcgcctcccacctgcgcaagctgcgtaagcggctcctccgc gatgccgatgacctgcagaagc/tgc[158]ctggcagtgtaccaggccggggcccgcgagggcgccgagcgcggcctca gcgccatccgcgagcgcctg - 3'

APPENDIX D: REGION OF *APOE* PROMOTER AND PRIMERS

APOE promoter

Sense Primer	5'-GGG GCT CCC CTG TGC TCA AG- 3'
Anti- Sense Primer	5'- TGT TCT CCC CCT GCC CCA GG- 3'

Region of APOE promoter covered

5'-

ggggctcccctgtgctcaaggtcacaaccaaagaggaagctgtgattaaaacccaggtcccatttgcaaagcctcgacttttagcaggtgcatcatactgttcccacccctcccatcccacttctgtccagccgcctagccccactttctttttttttctttttttgagacagtctccctcttgctgaggctggagtgcagtggcgagatctcggctcactgtaacctccgcctcccgggttcaagcgattctcctgcctcagcctcccaagtagctaggattacaggcgcccgccaccacgcctggctaacttttgtatttttagtagagatgggggtttcaccatgttggccaggctggtctcaaa[a/t]$^{-491}$ctcctgaccttaagtgattcgcccactgtggcctcccaaagtgctgggattacaggcgtgagc[t/c]$^{-427}$accgccccccagcccctcccatcccacttctgtccagccccctagccctactttctttctgggatccaggagtccagatccccagcccccctctccagattacattcatccaggcacaggaaaggacagggtcaggaaaggaggactctgggcggcagcctccacattccccttccacgcttggcccccagaatggaggagggtgtctg[t/g]$^{-219}$attactgggcgaggtgtcctcccttcctggggactgtgggggggtggtcaaaagacctctatgccccacctccttcctccctctgccctgctgtgcctggggcaggggggagaaca- 3'

APPENDIX E: LIST OF PROBES

SNP	ASSAY	PROBES (VIC/FAM)
rs4680	C__25746809_50	CCAGCGGATGGTGGATTTCGCTGGC[**A/G**]TGAAGGACAAGGTGTGCATGCCTGA
rs1800497	C__7486676_10	CACAGCCATCCTCAAAGTGCTGGTC[**T/C**]AGGCAGGCGCCCAGCTGGACGTCCA
rs6280	C__949770_10	GCCCCACAGGTGTAGTTCAGGTGGC[**T/C**]ACTCAGCTGGCTCAGAGATGCCATA
rs6265	C__11592758_10	TCCTCATCCAACAGCTCTTCTATCA[**G/A**]GTGTTCGAAAGTGTCAGCCAATGAT
rs36119840	C__648651_20	TCTGGGTTGGCAGCTGCAGCCTGCC[**G/A**]ATTCCGCTCTCTTCTAGGAAGCACT
rs1800169	C__7511603_10	AGATGTGGTGTTTTCCTGTATCCTC[**G/A**]GCCAGGTGAAGCATCAGGGCCTGAA

APPENDIX F: EVALUATION FORM FOR DISABILITY RATING SCALE

DISABILITY RATING SCALE

Patient Name:

Date of Rating:

Name of Person Completing Form:

A EYE OPENING

- ☐ (0) Spontaneous
- ☐ (1) To Speech
- ☐ (2) To Pain
- ☐ (3) None

0-SPONTANEOUS: eyes open with sleep/wake rhythms indicating active arousal mechanisms, does not assume awareness.
1-TO SPEECH AND/OR SENSORY STIMULATION: a response to any verbal approach, whether spoken or shouted, not necessarily the command to open the eyes. Also, response to touch, mild pressure.
2-TO PAIN: tested by a painful stimulus.
3-NONE: no eye opening even to painful stimulation.

B. COMMUNICATION ABILITY

- ☐ (0) Oriented
- ☐ (1) Confused
- ☐ (2) Inappropriate
- ☐ (3) Incomprehensible
- ☐ (4) None

0-ORIENTED: implies awareness of self and the environment. Patient able to tell you a) who he is; b) where he is; c) why he is there; d) year; e) season; f) month; g) day; h) time of day.
1-CONFUSED: attention can be held and patient responds to questions but responses are delayed and/or indicate varying degrees of disorientation and confusion.
2-INAPPROPRIATE: intelligible articulation but speech is used only in an exclamatory or random way (such as shouting and swearing); no sustained communication exchange is possible.
3-INCOMPREHENSIBLE: moaning, groaning or sounds without recognizable words, no consistent communication signs.
4-NONE: no sounds or communications signs from patient.

C. MOTOR RESPONSE

- ☐ (0) Obeying
- ☐ (1) Localizing
- ☐ (2) Withdrawing
- ☐ (3) Flexing
- ☐ (4) Extending

0-OBEYING: obeying command to move finger on best side. If no response or not suitable try another command such as “move lips,” “blink eyes,” etc. Do not include grasp or other reflex responses.
1-LOCALIZING: a painful stimulus at more than one site causes limb to move (even slightly) in an attempt to remove it. It is a deliberate motor act to move away from or remove the source of noxious stimulation. If there is doubt as to whether withdrawal or localization has occurred after 3 or 4 painful stimulations, rate as localization.
2-WITHDRAWING: any generalized movement away from a noxious stimulus that is more than a simple reflex response
3-FLEXING: painful stimulation results in either flexion at the elbow, rapid withdrawal with abduction of the shoulder or a slow withdrawal with adduction of the shoulder. If there is confusion between flexing and withdrawing, then use pinprick on hands.
4-EXTENDING: painful stimulation results in extension of the limb.
5-NONE: no response can be elicited. Usually associated with hypotonia. Exclude spinal transection as an explanation of lack of response; be satisfied that an adequate stimulus has been applied.

D. FEEDING (COGNITIVE ABILITY ONLY)

- ☐ (0.0) Complete
- ☐ (1.0) Partial
- ☐ (2.0) Minimal
- ☐ (3.0) None

Does the patient show awareness of how and when to perform this activity? Ignore motor disabilities that interfere with carrying out this function. (This is rated under Level of Functioning described below.)
0-COMPLETE: continuously shows awareness that he knows how to feed and can convey unambiguous information that he knows when this activity should occur.
1-PARTIAL: intermittently shows awareness that he knows how to feed and/or can intermittently convey reasonably clearly information that he knows when the activity should occur.
2-MINIMAL: shows questionable or infrequent awareness that he knows in a primitive way how to feed and/or shows infrequently by certain signs, sounds, or activities that he is vaguely aware when the activity should occur.
3-NONE: shows virtually no awareness at any time that he knows how to feed and cannot convey information by signs, sounds, or activity that he knows when the activity should occur.

E. TOILETING (COGNITIVE ABILITY ONLY)

- ☐ (0.0) Complete
- ☐ (1.0) Partial
- ☐ (2.0) Minimal
- ☐ (3.0) None

Does the patient show awareness of how and when to perform this activity? Ignore motor disabilities that interfere with carrying out this function. (This is rated under Level of Functioning described below.)
Rate best response for toileting based on bowel and bladder behavior
0-COMPLETE: continuously shows awareness that he knows how to toilet and can convey unambiguous information that he knows when this activity should occur.
1-PARTIAL: intermittently shows awareness that he knows how to toilet and/or can intermittently convey reasonably clearly information that he knows when the activity should occur.
2-MINIMAL: shows questionable or infrequent awareness that he knows in a primitive way how to toilet and/or shows infrequently by certain signs, sounds, or activities that he is vaguely aware when the activity should occur.
3-NONE: shows virtually no awareness at any time that he knows how to toilet and cannot convey information by signs, sounds, or activity that he knows when the activity should occur.

F. GROOMING (COGNITIVE ABILITY ONLY)

- ☐ (0.0) Complete
- ☐ (1.0) Partial
- ☐ (2.0) Minimal
- ☐ (3.0) None

Does the patient show awareness of how and when to perform this activity? Ignore motor disabilities that interfere with carrying out this function. (This is rated under Level of Functioning described below.) Grooming refers to bathing, washing, brushing of teeth, shaving, combing or brushing of hair and dressing.
0-COMPLETE: continuously shows awareness that he knows how to groom self and can convey unambiguous information that he knows when this activity should occur.
1-PARTIAL: intermittently shows awareness that he knows how to groom self and/or can intermittently convey reasonably clearly information that he knows when the activity should occur.
2-MINIMAL: shows questionable or infrequent awareness that he knows in a primitive way how to groom self and/or shows infrequently by certain signs, sounds, or activities that he is vaguely aware when the activity should occur.
3-NONE: shows virtually no awareness at any time that he knows how to groom self and cannot convey information by signs, sounds, or activity that he knows when the activity should occur.

G. LEVEL OF FUNCTIONING (PHYSICAL, MENTAL, EMOTIONAL OR SOCIAL FUNCTION)

- ☐ (0.0) Completely Independent
- ☐ (1.0) Independent in special environment
- ☐ (2.0) Mildly Dependent-Limited assistance (non-resid - helper)
- ☐ (3.0) Moderately Dependent-moderate assist (person in home)
- ☐ (4.0) markedly Dependent-assist all major activities, all times
- ☐ (5.0) Totally Dependent-24 hour nursing care

0-COMPLETELY INDEPENDENT: able to live as he wishes, requiring no restriction due to physical, mental, emotional or social problems.
1-INDEPENDENT IN SPECIAL ENVIRONMENT: capable of functioning independently when needed requirements are met (mechanical aids)
2-MILDLY DEPENDENT: able to care for most of own needs but requires limited assistance due to physical, cognitive and/or emotional problems (e.g., needs non-resident helper).
3-MODERATELY DEPENDENT: able to care for self partially but needs another person at all times. (person in home)
4-MARKEDLY DEPENDENT: needs help with all major activities and the assistance of another person at all times.
5-TOTALLY DEPENDENT: not able to assist in own care and requires 24-hour nursing care

H. EMPLOYABILITY (AS A FULL TIME WORKER, HOMEMAKER OR STUDENT)

- ☐ (0.0) Not Restricted
- ☐ (1.0) Selected jobs, competitive
- ☐ (2.0) Sheltered workshop, Non-competitive
- ☐ (3.0) Not Employable

0-NOT RESTRICTED: can compete in the open market for a relatively wide range of jobs commensurate with existing skills; or can initiate, plan execute and assume responsibilities associated with homemaking; or can understand and carry out most age relevant school assignments.
1-SELECTED JOBS, COMPETITIVE: can compete in a limited job market for a relatively narrow range of jobs because of limitations of the type described above and/or because of some physical limitations; or can initiate, plan, execute and assume many but not all responsibilities associated with homemaking; or can understand and carry out many but not all school assignments.
2-SHELTERED WORKSHOP, NON-COMPETITIVE: cannot compete successfully in a job market because of limitations described above and/or because of moderate or severe physical limitations; or cannot without major assistance initiate, plan, execute and assume responsibilities for homemaking; or cannot understand and carry out even relatively simple school assignments without assistance.
3-NOT EMPLOYABLE: completely unemployable because of extreme psychosocial limitations of the type described above, or completely unable to initiate, plan, execute and assume any responsibilities associated with homemaking; or cannot understand or carry out any school assignments.

Notes:

The psychosocial adaptability or "employability" item takes into account overall cognitive and physical ability to be an employee, homemaker or student.

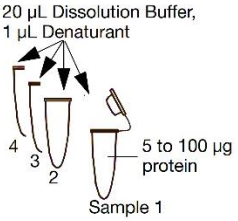
This determination should take into account considerations such as the following:

1. Able to understand, remember and follow instructions.
2. Can plan and carry out tasks at least at the level of an office clerk or in simple routine, repetitive industrial situation or can do school assignments.
3. Ability to remain oriented, relevant and appropriate in work and other psychosocial situations.
4. Ability to get to and from work or shopping centers using private or public transportation effectively.
5. Ability to deal with number concepts.
6. Ability to make purchases and handle simple money exchange problems.
7. Ability to keep track of time and appointments.

APPENDIX G: iTRAQ REAGENT LABELLING PROTOCOL

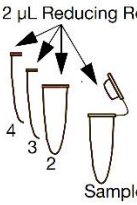
A Reduce the Proteins and Block Cysteine

1a. To each of up to four sample tubes, add 20 µL Dissolution Buffer and 1 µL Denaturant.



b. Vortex to mix.

2a. To each sample tube, add 2 µL Reducing Reagent.

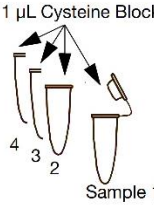


b. Vortex to mix, then spin.

c. Incubate the tubes at 60 °C for 1 hour.

d. Spin to bring the sample to the bottom of the tube.

3a. To each tube, add 1 µL Cysteine Blocking Reagent.

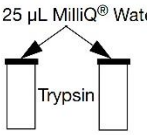


b. Vortex to mix, then spin.

c. Incubate the tubes at room temperature for 10 minutes.

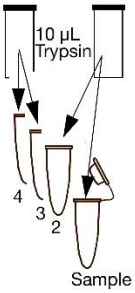
B Digest the Proteins with Trypsin

1a. Reconstitute a vial of trypsin with 25 µL MilliQ® Water or equivalent. (For three or four samples, reconstitute two vials.)



b. Vortex to mix, then spin.

2a. To each sample tube, add 10 µL of the trypsin solution.



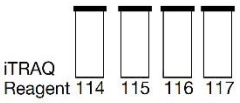
b. Vortex to mix, then spin.

c. Incubate at 37 °C overnight (12 to 16 hours).

d. Spin to bring the sample digest to the bottom of the tube.

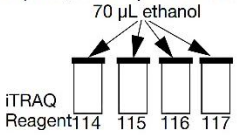
C Label the Protein Digests with iTRAQ® Reagents

1a. Allow iTRAQ™ reagent(s) required to reach room temperature.



b. Spin to bring the solution to the bottom of the tube.

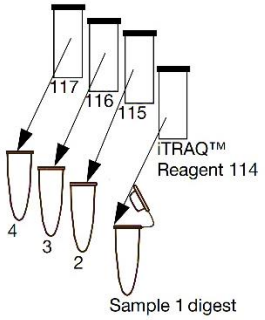
2a. To each iTRAQ™ reagent required, add 70 µL of ethanol.



b. Vortex to mix, then spin.

C Label the Protein Digests with iTRAQ® Reagents (*continued*)

3a. Transfer the contents of one iTRAQ® Reagent vial to one sample tube.

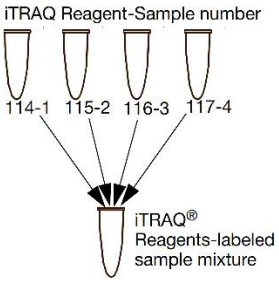


b. Vortex to mix, then spin.

c. Incubate at room temperature for 1 hour.

D Combining the iTRAQ® Reagent-Labeled Digests for Analysis

1a. Combine the contents of each iTRAQ® Reagent-labeled sample tube in a fresh tube.+



b. Vortex to mix, then spin.

IMPORTANT! Before LC/MS/MS analysis, clean up the sample mixture using cation exchange. If the sample mixture is complex, clean up and fractionate using high-resolution cation exchange.

+(*Optional*) Before combining the samples, reduce the organic concentration, then clean up an aliquot of each labeled sample digest using a ZipTip®. Analyze each aliquot by MS/MS to verify that you see peaks at the m/z of the appropriate iTRAQ® Reagent reporter group. If not, relabel the protein digest.

APPENDIX H: LIST OF 83 PROTEINS IDENTIFIED BY iTRAQ

	Accession	Name	Molecular Function	Subcellular location
1	sp|P0C0L5|CO4B_HUMAN	Complement C4-B	Carbohydrate binding, endopeptidase inhibitor activity, complement binding	Secreted
2	sp|P04114|APOB_HUMAN	Apolipoprotein B-100	Cholesterol transporter activity, lipase binding, low-density lipoprotein particle receptor binding, phospholipid binding, heparin binding	Cytoplasm, secreted
3	sp|P01023|A2MG_HUMAN	Alpha-2-macroglobulin	Calcium- dependent protein binding, growth factor binding, interleukin-8 binding, receptor binding, serine-type endopeptidase inhibitor activity, tumor necrosis factor binding, enzyme binding, interleukin-1 binding, protease binding	Secreted
4	sp|P00751|CFAB_HUMAN	Complement factor B	Complement binding	Secreted
5	sp|P02452|CO1A1_HUMAN	Collagen alpha-1(I) chain	Extracellular matrix structural constituent, identical protein binding, platelet-derived growth factor binding, metal ion binding	Secreted › extracellular space › extracellular matrix
6	sp|Q14624|ITIH4_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H4	Endopeptidase inhibitor activity, serine-type endopeptidase inhibitor activity	Secreted
7	sp|P00450|CERU_HUMAN	Ceruloplasmin	Chaperone binding, ferroxidase activity, copper ion binding	Secreted
8	sp|P02751|FINC_HUMAN	Fibronectin	Collagen binding, integrin binding, protease binding, heparin binding, peptidase activator activity	Secreted › extracellular space › extracellular

9	sp|P06727|APOA4_HUMAN	Apolipoprotein A-IV	Antioxidant activity, copper ion binding, lipid transporter activity, phosphatidylcholine-sterol-O-acyltransferase activator activity, protein homodimerization activity, cholesterol transporter activity, lipid binding, phosphatidylcholine binding	Secreted
10	sp|P19823|ITIH2_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H2	Endopeptidase inhibitor activity, serine-type endopeptidase inhibitor activity	Secreted
11	sp|P02790|HEMO_HUMAN	Hemopexin	Heme transporter activity, metal ion binding	Secreted
12	sp|P08603|CFAH_HUMAN	Complement factor H	Heparan sulfate proteoglycan binding, heparin binding	Secreted
13	sp|P02750|A2GL_HUMAN	Leucine-rich alpha-2-glycoprotein	Brown fat cell differentiation, positive regulation of endothelial cell proliferation, positive regulation of transforming growth factor beta receptor signaling pathway, positive regulation of angiogenesis	Secreted
14	sp|P01011|AACT_HUMAN	Alpha-1-antichymotrypsin	DNA binding, serine-type endopeptidase inhibitor activity	Secreted
15	sp|P04217|A1BG_HUMAN	Alpha-1B-glycoprotein	Unknown	Secreted
16	sp|P19827|ITIH1_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H1	Calcium ion binding, serine-type endopeptidase inhibitor activity	Secreted
17	sp|P01042|KNG1_HUMAN	Kininogen-1	Cysteine-type endopeptidase inhibitor activity, heparin Binding, zinc ion binding, receptor binding	Secreted › extracellular space
18	sp|P01008|ANT3_HUMAN	Antithrombin-III	Heparin binding, serine-type endopeptidase inhibitor activity, protease binding	Secreted › extracellular space

19	sp|P02647|APOA1_HUMAN	Apolipoprotein A-I	Apolipoprotein A-I receptor binding, apolipoprotein receptor binding, beta-amyloid binding, chemorepellent activity, cholesterol binding, cholesterol transporter activity, enzyme binding, high-density lipoprotein particle binding, high-density lipoprotein particle receptor binding, identical protein binding, lipase inhibitor activity, phosphatidylcholine-sterol O-acyltransferase activator activity, phospholipid binding, phospholipid transporter activity	Secreted
20	sp|P02765|FETUA_HUMAN	Alpha-2-HS-glycoprotein	Cysteine-type endopeptidase inhibitor activity, kinase inhibitor activity	Secreted
21	sp|P06396|GELS_HUMAN	Gelsolin	Calcium ion binding	Secreted, Cytoskeleton
22	sp|P25311|ZA2G_HUMAN	Zinc-alpha-2-glycoprotein	Glycoprotein binding, protein transmembrane transporter activity, ribonuclease activity, peptide antigen binding	Secreted
23	sp|P08123|CO1A2_HUMAN	Collagen alpha-2(I) chain	Extracellular matrix structural constituent, identical protein binding, platelet-derived growth factor binding, metal ion binding, protein binding, bridging	Secreted › extracellular space › extracellular matrix
24	sp|P04196|HRG_HUMAN	Histidine-rich glycoprotein	Cysteine-type endopeptidase inhibitor activity, heme binding, heparan sulfate proteoglycan binding, heparin binding, metal ion binding, serine-type endopeptidase inhibitor activity, zinc ion binding, immunoglobulin binding, receptor binding	Secreted
25	sp|P05155|IC1_HUMAN	Plasma protease C1 inhibitor	Serine-type endopeptidase inhibitor activity	Secreted
26	sp|P00734|THRB_HUMAN	Prothrombin	Calcium ion binding, receptor binding, thrombospondin receptor activity, growth factor activity, serine-type endopeptidase activity	Secreted › extracellular space
27	sp|P10909|CLUS_HUMAN	Clusterin	Misfolded protein binding, ubiquitin protein ligase binding	Secreted, cytoplasmic or nuclear component

28	sp|P02749|APOH_HUMAN	Beta-2-glycoprotein 1	Glycoprotein binding, identical protein binding, lipoprotein lipase activator activity, heparin binding, lipid binding, phospholipid binding	Secreted
29	sp|P04004|VTNC_HUMAN	Vitronectin	Extracellular matrix binding, integrin binding, scavenger receptor activity, heparin binding, polysaccharide binding	Secreted › extracellular space
30	sp|P36955|PEDF_HUMAN	Pigment epithelium-derived factor	Serine-type endopeptidase inhibitor activity	Secreted, melanosome
31	sp|P00747|PLMN_HUMAN	Plasminogen	Apolipoprotein binding, serine-type endopeptidase activity, protein domain specific binding, serine-type peptidase activity	Secreted
32	sp|P02774|VTDB_HUMAN	Vitamin D-binding protein	Actin binding, vitamin D binding, calcidiol binding, vitamin transporter activity	Secreted
33	sp|P51884|LUM_HUMAN	Lumican	Collagen binding, extracellular matrix structural constituent	Secreted › extracellular space › extracellular matrix
34	sp|P43652|AFAM_HUMAN	Afamin	Vitamin E binding	Secreted
35	sp|P02748|CO9_HUMAN	Complement component C9	Complement activation, alternative pathway	Secreted. Cell membrane; Multi-pass membrane protein
36	sp|P02649|APOE_HUMAN	Apolipoprotein E	Antioxidant activity, cholesterol transporter activity, heparin binding, hydroxyapatite binding, lipid transporter activity, lipoprotein particle binding, low-density lipoprotein particle receptor binding, metal chelating activity, phosphatidylcholine-sterol O-acyltransferase activator activity, phospholipid binding, very-low-density lipoprotein particle receptor binding	Secreted

37	sp|P09871|C1S_HUMAN	Complement C1s subcomponent	Calcium ion binding, serine-type endopeptidase activity, identical protein binding	Unknown
38	sp|P08697|A2AP_HUMAN	Alpha-2-antiplasmin	Endopeptidase inhibitor activity, protein homodimerization activity, serine-type endopeptidase inhibitor activity, protease binding	Secreted
39	sp|P01871|IGHM_HUMAN	Ig mu chain C region	Antigen binding	Secreted, cell membrane
40	sp|Q96PD5|PGRP2_HUMAN	N-acetylmuramoyl-L-alanine amidase	N-acetylmuramoyl-L-alanine amidase activity, peptidoglycan binding, zinc ion binding, peptidoglycan receptor activity	Secreted, membrane
41	sp|P06681|CO2_HUMAN	Complement C2	Metal ion binding, serine-type endopeptidase activity	
42	sp|P35858|ALS_HUMAN	Insulin-like growth factor-binding protein complex acid labile subunit	Insulin-like growth factor binding	Secreted › extracellular space
43	sp|P02652|APOA2_HUMAN	Apolipoprotein A-II	Apolipoprotein receptor binding, cholesterol transporter activity, high-density lipoprotein particle binding, high-density lipoprotein particle receptor binding, lipase inhibitor activity, lipid transporter activity, phosphatidylcholine-sterol O-acyltransferase activator activity, phospholipid binding, protein homodimerization activity, cholesterol binding, lipid binding, phosphatidylcholine binding, protein heterodimerization activity	Secreted
44	sp|P13671|CO6_HUMAN	Complement component C6	Complement activation, classical pathway	Secreted
45	sp|P01031|CO5_HUMAN	Complement C5	Chemokine activity, receptor binding, endopeptidase inhibitor activity	Secreted
46	sp|P01019|ANGT_HUMAN	Angiotensinogen	Growth factor activity, serine-type endopeptidase inhibitor activity, superoxide-generating NADPH oxidase activator activity, type 1 angiotensin receptor binding, hormone activity, type 2 angiotensin receptor binding	Secreted

47	sp|P02741|CRP_HUMAN	C-reactive protein	Calcium ion binding, choline binding, complement component C1q binding, low-density lipoprotein particle binding, cholesterol binding	Secreted
48	sp|P05156|CFAI_HUMAN	Complement factor I	Metal ion binding, serine-type endopeptidase activity, scavenger receptor activity	Secreted › extracellular space
49	sp|P00738|HPT_HUMAN	Haptoglobin	Antioxidant activity, serine-type endopeptidase activity, hemoglobin binding	Secreted
50	sp|P02753|RET4_HUMAN	Retinol-binding protein 4	Retinal binding, retinol transporter activity, retinol binding	Secreted
51	sp|P0DJI8|SAA1_HUMAN	Serum amyloid A-1 protein	G-protein coupled receptor binding, heparin binding	Secreted
52	sp|P02768|ALBU_HUMAN	Serum albumin	Antioxidant activity, copper ion binding, drug binding, pyridoxal phosphate binding, zinc ion binding, chaperone binding, DNA binding, fatty acid binding, toxic substance binding	Secreted
53	sp|P02760|AMBP_HUMAN	Protein AMBP	Calcium channel inhibitor activity, heme binding, protein homodimerization activity, serine-type endopeptidase inhibitor activity, small molecule binding, calcium oxalate binding, IgA binding	Secreted
54	sp|P00748|FA12_HUMAN	Coagulation factor XII	Misfolded protein binding, serine-type endopeptidase activity, serine-type aminopeptidase activity	Secreted
55	sp|P05546|HEP2_HUMAN	Heparin cofactor 2	Endopeptidase inhibitor activity, serine-type endopeptidase inhibitor activity, heparin binding	Secreted › extracellular space
56	sp|P07477|TRY1_HUMAN	Trypsin-1	Metal ion binding, serine-type endopeptidase activity	Secreted › extracellular space
57	sp|P02775|CXCL7_HUMAN	Platelet basic protein	Glucose transmembrane transporter activity	Secreted
58	sp|P05452|TETN_HUMAN	Tetranectin	Calcium ion binding, heparin binding, carbohydrate binding, kringle domain binding	Secreted

59	sp|P02747|C1QC_HUMAN	Complement C1q subcomponent subunit C	Complement activation, classical pathway	Secreted
60	sp|P18428|LBP_HUMAN	Lipopolysaccharide-binding protein	Lipopolysaccharide binding, receptor binding, lipoteichoic acid binding	Secreted
61	sp|P00736|C1R_HUMAN	Complement C1r subcomponent	Calcium ion binding, serine-type peptidase activity, serine-type endopeptidase activity	Unknown
62	sp|P01024|CO3_HUMAN	Complement C3	C5L2 anaphylatoxin chemotactic receptor binding, endopeptidase inhibitor activity, receptor binding	Secreted
63	sp|P02745|C1QA_HUMAN	Complement C1q subcomponent subunit A	Cell-cell signaling, complement activation, classical pathway	Secreted
64	sp|P68871|HBB_HUMAN	Hemoglobin subunit beta	Heme binding, iron ion binding, oxygen transporter activity, hemoglobin binding, oxygen binding	Cytosol
65	sp|Q06033|ITIH3_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H3	Endopeptidase inhibitor activity, serine-type endopeptidase inhibitor activity	Secreted
66	sp|P29622|KAIN_HUMAN	Kallistatin	Serine-type endopeptidase inhibitor activity	Secreted
67	sp|P02743|SAMP_HUMAN	Serum amyloid P-component	Calcium ion binding, complement component C1q binding, unfolded protein binding, carbohydrate binding, virion binding	Secreted
68	sp|P07225|PROS_HUMAN	Vitamin K-dependent protein S	Calcium ion binding, endopeptidase inhibitor activity	Secreted
69	sp|P02671|FIBA_HUMAN	Fibrinogen alpha chain	Structural molecule activity	Secreted
70	sp|P03952|KLKB1_HUMAN	Plasma kallikrein	Serine-type endopeptidase activity	Secreted
71	sp|P69905|HBA_HUMAN	Hemoglobin subunit alpha	Heme binding, oxygen binding, iron ion binding, oxygen transporter activity	Cytosol
72	sp|O75636|FCN3_HUMAN	Ficolin-3	Carbohydrate binding	Secreted
73	sp|P07357|CO8A_HUMAN	Complement component C8 alpha chain	Complement activation, alternative pathway	Secreted, cell membrane
74	sp|P22792|CPN2_HUMAN	Carboxypeptidase N subunit 2	Enzyme regulator activity	Secreted

75	sp|P68133|ACTS_HUMAN	Actin, alpha skeletal muscle	ADP binding, myosin binding, structural constituent of cytoskeleton, ATP binding	Cytoplasm › cytoskeleton
76	sp|P07358|CO8B_HUMAN	Complement component C8 beta chain	Complement activation, alternative pathway	Secreted
77	sp|Q6UXB8|PI16_HUMAN	Peptidase inhibitor 16	Peptidase inhibitor activity	Membrane
78	sp|P26927|HGFL_HUMAN	Hepatocyte growth factor-like protein	Serine-type endopeptidase activity	Secreted
79	sp|P02654|APOC1_HUMAN	Apolipoprotein C-I	Fatty acid binding, phosphatidylcholine binding, phosphatidylcholine-sterol O-acyltransferase activator activity, phospholipase inhibitor activity, lipase inhibitor activity	Secreted
80	sp|P05543|THBG_HUMAN	Thyroxine-binding globulin	Hormone binding, serine-type endopeptidase inhibitor activity	Secreted
81	sp|P32119|PRDX2_HUMAN	Peroxiredoxin-2	Antioxidant activity, thioredoxin peroxidase activity	Cytoplasm
82	sp|P02746|C1QB_HUMAN	Complement C1q subcomponent subunit B	Complement activation, classical pathway	Secreted
83	sp|P00740|FA9_HUMAN	Coagulation factor IX	Calcium ion binding, serine-type endopeptidase activity	Secreted